PATENT
Docket No. 204372000320

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Nancy J. Robins
NANCY J. ROBINS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#9
RD

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF LYNN E. SPITLER PURSUANT TO 37 C.F.R § 1.132

Box AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Lynn E. Spitler, declare as follows:

1. I am a coinventor in regard to the above-referenced patent application, and have been supervising clinical trials using antitumor vaccines which contain recombinant human prostate-specific antigen (PSA) as the active ingredient. I am an experienced immunologist and medical doctor. A copy of my *curriculum vitae* is attached hereto as Exhibit A.

2. I note that The Examiner makes the point several times that previous attempts to actively immunize patients with prostate adenocarcinoma cells admixed with adjuvant have shown little or no therapeutic benefit. However, the use of whole tumor cells is not analogous to the use of recombinant protein such as purified PSA. Whole PSA is not represented on the surface of the

tumor cells; thus, the patients would not be expected to be effectively immunized to PSA via this approach. PSA is synthesized within the tumor cells and secreted; therefore, the patients' immune system might be exposed to small amounts of PSA through this approach as some of the tumor cells die and release the internal PSA; these small amounts of antigen would be presented to the immune system in the context of all the other antigens present on and in the tumor cells. This would not be likely to result in an immune response to the PSA. Peptides derived from PSA are present on the surface of the tumor cells, presented in the context of HLA molecules. For these to induce an immune response, it would be expected that they would have to be taken up by the professional antigen presenting cells and represented on the surface of these cells. Again, this would be occurring in the presence of all the other antigens present on and in the tumor cells.

3. Thus, one cannot take failure of the approaches using whole tumor cells to indicate that immunization with specific antigens will fail (including antigens overrepresented in the prostate gland, an immunologically effective portion thereof, or an antiidiotypic antibody). Indeed, it is the recognition that the use of pure antigens may represent a more effective means of immunization for cancer therapy which has led to intense activity in this field and numerous clinical trials (Spitler, L.E., Engineered Vaccines for Cancer, *Sixth International Congress on Anti-Cancer Treatment* (1995) Paris, February 6-9, 1996; Spitler, L.E., Cancer Vaccines: The Interferon Analogy, *Cancer Biotherapy* (1995) 10:1-3 (copies attached).

4. Clinical trials in a number of patients have been initiated using recombinantly produced human PSA. PSA is a well known glycoprotein with a molecular weight of 33-34 kDa. PSA was cloned, expressed, and produced by large scale suspension cultures of High Five™ insecT-cells infected with recombinant PSA-baculovirus. PSA has the amino acid sequence:

D L I V G G W E C E K H S Q P W Q V L V

A S R G R A V C G G V L V H P Q W V L T

A A H C I R N K S V I L L G R H S L F H

P E D T G Q V F Q V S H S F P H P L Y D

M S L L K N R F L R P G D D S S H D L M

L L R L S E P A E L T D A V K V M D L P

T Q E P A L G T T C Y A S G W G S I E P

E E F L T P K K L Q C V D L H V I S N D

V C A Q V H P Q K V T K F M L C A G R W

T G G K S T C S G D S G G P L V C N G V

L Q G IT S W G S E Q C A L P E R P S L

Y T K V V H Y R K W I K D T I V A N P

PSA was purified from the culture supernatants by affinity chromatography using a monoclonal antibody specific to PSA and incorporated into liposomes of the following composition:

Each ml. (one dose) contained:

Component	Quantity (mg/ml)
Prostate Specific Antigen	Approximately 0.10
Monophosphoryl Lipid A	0.20
Dimyristoyl phosphatidylcholine	61.01
Dimyristoyl phosphatidylglycerol	6.89
Cholesterol	29.00
Polysorbate 80	0.10*

Buffer: 20 mM TRIS-glycine in 140 mM NaCl

*Maximum quantity that can be incorporated. The actual amount incorporated is unknown.

5. Six (6) patients were immunized with the prostate cancer vaccine described above. Each patient was given 1 ml of the vaccine intramuscularly, divided into 2 sites, on days 0, 30, and 60. An additional two (2) patients have been treated by intravenous administration of the product with the same dose and schedule of administration. All patients were carefully monitored for adverse effects through clinical and laboratory evaluation. No adverse event attributable to the vaccine was observed in any patient. Specifically, there were no adverse events suggesting an autoimmune reaction to cross-reacting antigens.

6. Immunologic tests of T and B cell responses were performed before each immunization and 2 weeks after each immunization. Evidence of T-cell immune responses was observed. (Harris, D.T., et al., Active Specific Immunization of Patients with Hormone-refractory Prostate Cancer using OncoVax-P™, ASCO Proceedings (1996)) (copy enclosed).

7. For immunologic testing of patients, a pool of peptides representing CTL epitopes of PSA was used:

Amino Acid Numbers	Sequence
29-37	VLVHPQWVL
98-106	MLLRLSEPA
141-150	FLTPKKLQCV
146-154	KLQCVDLHV
154-163	VISNDVCAQV

Peripheral blood mononuclear cells were harvested at the times indicated and incubated with the PSA peptide pool. On the third day of culture, Interleukin-2 (IL-2) was added. On day 7, the cultures were restimulated with autologous antigen presenting cells and the PSA peptide pool. The cultures were assayed on day 19 to determine the levels of gamma interferon and Interleukin-4 (IL-4) production. Results in the first four patients studied showed an increase in the production of these cytokines in some of the samples after immunization, as compared to before immunization, thus indicating a T-cell response. These results are shown in Exhibit B.

8. The foregoing results show that in clinical trials, the vaccine of the invention causes no adverse side effects sufficient to undermine its efficacy and that the vaccine is capable of eliciting an immune response to the PSA antigen mediated by T-cells.

9. In more detail, in regard to safety, there were no local reactions at the injection site, no symptoms of prostatitis, no signs of autoimmune disease, no malaise or fevers, and no signs of allergic reactions.

10. All of the patients had metastatic disease, had failed hormonal therapy, and had rising levels of PSA at the time of entry into the study.

11. As shown in the table below, and in Exhibit B, two of the six patients (patients no. 2 and no. 3) had immulogical responses to PSA and three others had some suggestion of reaction (patients no. 1, no. 4 and no. 5). Lymphocytes from patient no. 2 showed proliferation to PSA and to PSA peptides as well as production of the cytokines γ-interferon and interleukin-4 in response to PSA peptides. The lymphocytes from patient no. 3 showed proliferation in response to PSA in two separate tests and this patient had a positive skin response to PSA. We were not able to measure CTLs directly because the assay is still under development; however, the cytokine production in response to PSA peptide stimulation shown in two patients is correlated with CTL development. The following table summarizes the results obtained. N.T. refers to not tested.

Immulogic Responses Summary							
Patient #	PSA Skin Test	Lympho #1 PSA	Lympho #2 PSA	Lympho Peptide	Cytokine Gamma IFN	Cytokine IL-4	Antibody
1. AW	-	-	-	+/-	-	+	0
2. JH	N.T.	+	-	+	+	+	0
3. MD	+	+	+	-	-	+/-	+/-
4. MED	-	N.T.	-	-	+/-	-	0
5. HN	-	N.T.	+/-	-	N.T.	N.T.	0
6. JLB	-	N.T.	-	-	N.T.	N.T.	0

12. Exhibit C contains copies of overhead transparencies prepared for formal presentation of results of the clinical study.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at Tiburon, California on

11/1/96 Lynn E Spit

Date Lynn E. Spitler

CURRICULUM VITAE

LYNN E. SPITLER, M.D.

1895 Mountain View Drive
Tiburon, California 94920

(415) 435-9861
FAX (415) 435-6851

Social Security #366-38-1697

Education:

University of Michigan, Ann Arbor, Michigan	1956-1959
University of Michigan Medical School Ann Arbor, Michigan M.D. (cum laude)	1959-1963

Training:

Intern	Highland Alameda County Hospital Oakland, California	1963-1964
Resident	University of California School of Medicine San Francisco, California	1964-1966
Research Fellow	H.S. Lawrence, M.D. Department of Medicine - Immunology New York University New York, New York	1966-1967
Immunology Trainee	H. Hugh Fudenberg, M.D. University of California School of Medicine San Francisco, California	1967-1969

EXHIBIT A

Teaching Appointments and Employment:

Instructor of Medicine in Residence University of California School of Medicine San Francisco, California 94143	1970-1971
Assistant Professor of Medicine in Residence University of California School of Medicine San Francisco, California 94143	1971-present
Research Associate Cancer Research Institute University of California School of Medicine San Francisco, CA 94143	1971-1978
Director, Melanoma Center Northern California Health Center San Francisco, California 94118	1978-1990
Director of Research Children's Hospital of San Francisco San Francisco, California 94118	1978-1981
Member, Graduate Group in Comparative Pathology Department of Comparative Pathology University of California, Davis Davis, California	1976-1981
Senior Vice President XOMA Corporation 2910 Seventh Street Berkeley, California 94710	1981-1988
Associate Scientific Director Biotherapeutics, Inc. 357 Riverside Drive Franklin, Tennessee, 37065-1676	1988-1989

Director — 1990-present
Northern California Melanoma Centers
1895 Mountain View Drive
Tiburon, California 94920

President — 1991-present
Jenner Technologies
1895 Moountain View Drive
Tiburon, California 94920

Awards and Honors:

Recipient: Dernham Senior Fellowship — 1969-1971
California Div. of the American Cancer Society

Recipient: Research Career Development Award — 1971-1976
National Institutes of Health

Alpha Omega Alpha (Junior year) — 1961

Outstanding Young Women of America — 1968

Who's Who of American Women — 1972

Who's Who in the West — 1973

Board Certification:

American Board of Internal Medicine — 1972
American Board of Allergy and Immunology — 1974

Licensure:

Michigan	25985
New York	96454
California	C-26446

Memberships in Professional Societies:

American Association of Immunologists
American Association for the Advancement of Science
Alpha Epsilon Iota
Western Society for Clinical Research
American Federation of Clinical Research
Society of Biological Therapy
American Association for Cancer Research

Patents:

Patent #4,489,810 for "Composition and Method for Transplantation Therapy"

Patent #4,590,071 for "Human Melanoma Specific Immunotoxins"

PUBLIC SERVICE

National Review Committees:

Allergy and Immunology Research Committee, NIAID, NIH	1976-1980
Merit Review Board in Immunology, VA, Washington, D.C. (Chairman 1979-1980)	1976-1980

Editorial Boards:

The Journal of Immunology	1975-1978
The International Journal of Immunopharmacology	1979-1984
Immunologia Clinica e Sperimentale	1982-1986
Antibody Immunoconjugates and Radiopharmaceuticals (Associate Editor)	1987-present
Molecular Biotherapy	1987-present
Cancer Biotherapy	1991-present

Manuscript Reviews:

American Review of Respiratory Disease
Annals of Allergy
Annals of Internal Medicine
Archives of Dermatology
Archives of Internal Medicine
California Medicine
Cancer
Cancer Immunology and Immunopathology
Cancer Research
Cellular Immunology
Chest
Infection and Immunity
Infectious Disease and Immunology
International Journal of Immunopharmacology
Journal of Clinical Investigation
Journal of Immunology
Journal of Infectious Diseases
Journal of the American Academy of Dermatology
Molecular Biotherapy
Nature
New England Journal of Medicine
Science
The Western Journal of Medicine

Special Consultant:

National Institutes of Health Grant Reviews
National Institutes of Health Site Visits
United States Tuberculosis Panel Task Group
Atomic Energy Commission Site Visits
Public Education Panel, National Multiple Sclerosis Society
Review of Grant Applications for the National Science Foundation
Enterprise for High School Students, Medical Apprenticeship Program, San Francisco, California
Board of Directors, San Francisco Unit, American Cancer Society
Research and Human Experimentation Committee, Children's Hospital of San Francisco
U.S. Energy Research Development Administration Site Visits

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LYNN E. SPITLER, M.D.

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Revised January, 1992

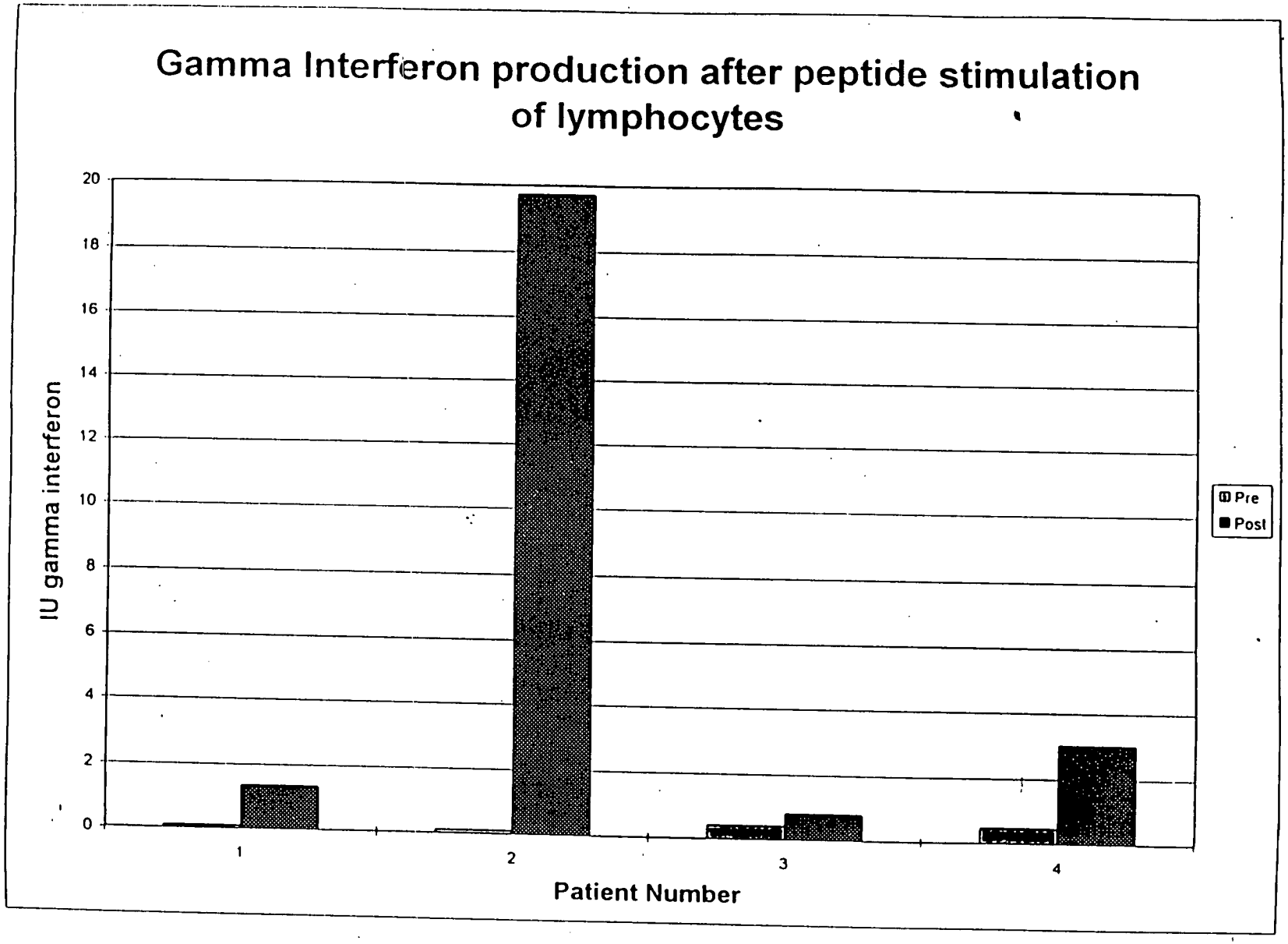
Gamma Interferon production after peptide stimulation of lymphocytes
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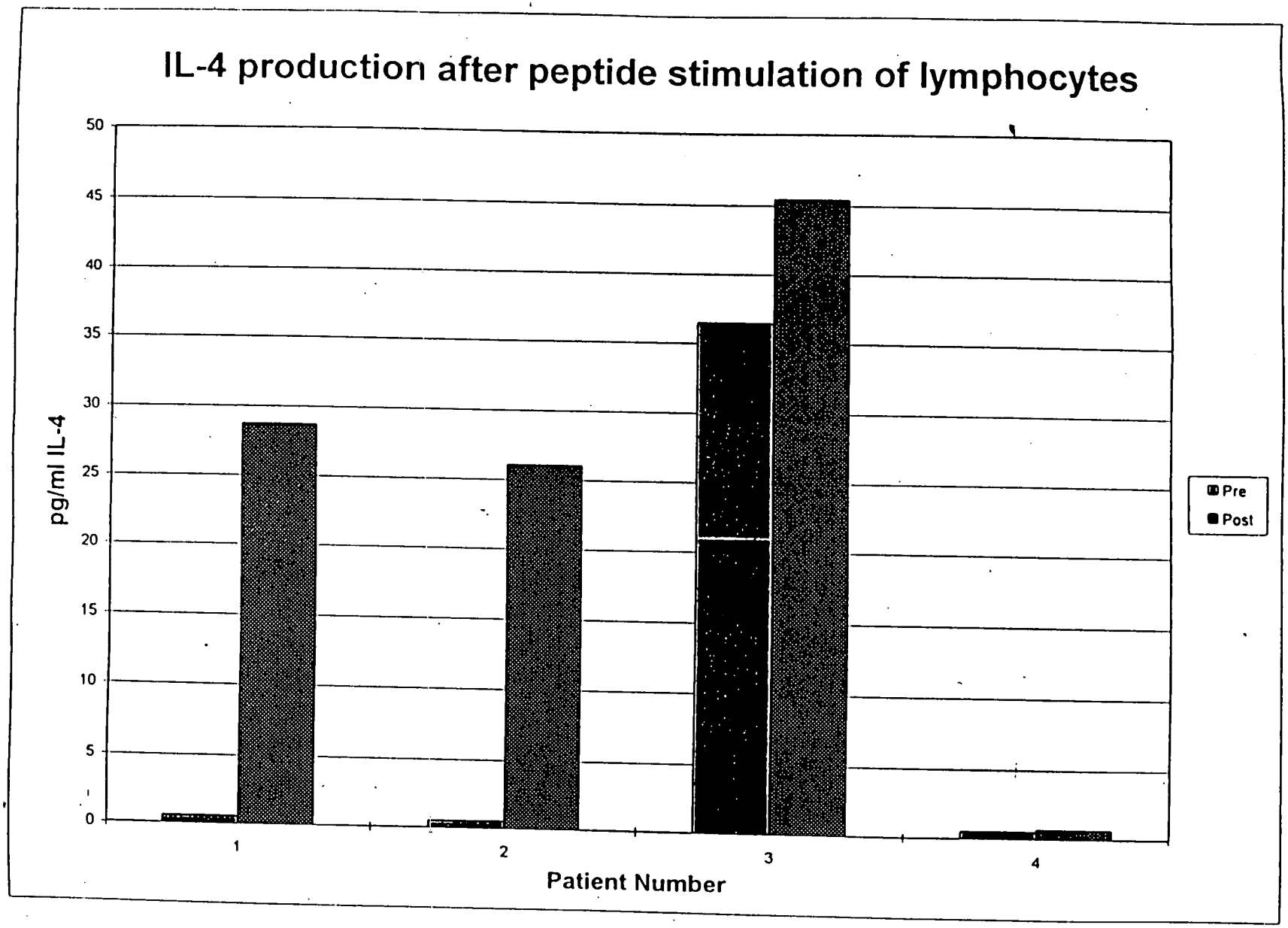
Exhibit B


IL-4 production after peptide stimulation of lymphocytes
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Pre
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Exhibit B

PATENT
Docket No. 204372000320

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being facsimile transmitted to the United States Patent and Trademark Office on November 4, 1996.

Nancy J. Robins

Nancy J. Robins

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF PHILIP O. LIVINGSTON, MD PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Philip O. Livingston, MD, declare as follows:

1. I am a Associate Professor and in charge of the laboratory of Developmental Tumor Vaccinology at Memorial Sloan-Ketter Cancer Center in New York, New York. I am a member of the Scientific Advisory Board for Jenner Technologies, the assignee of this application, and am also a shareholder in the company. A copy of my *Curriculum Vitae* is attached hereto as Exhibit A.

2. I have reviewed the Declaration Under 37 C.F.R. 1.132 prepared by Dr. Lynn E. Spitler describing the results of a clinical study directed to the use of prostate specific antigen

(PSA) as an active ingredient in an antiprostate cancer vaccine. I am also familiar with the study itself, and with the results that were obtained.

3. The purpose of the study was to obtain initial evidence the vaccines would raise a sufficient cellular immune response to have a beneficial effect with respect to prostate tumors. Such a result could be shown directly by measuring cytotoxic lymphocyte (CTL) generation; however, I am aware that this was not possible in these studies because of problems assaying cytotoxic T cell activity. This problem is widespread in the field, despite occasional reports to the contrary, and a lack of sensitive assays for CTL activity is widely considered to be one of the major obstacles to the development of a new generation of vaccines capable of inducing cytotoxic T cells against tumors. This is due to uncertainty over the optimal assay, the optimal time from immunization to blood drawing, and whether testing for CTL activity in the peripheral blood lymphocytes would ever be capable of reflecting the systemic induction of effective CTLs. Consequently, other assays for T cell immunity have been widely used..

4. The responses measured are understood in the art to be satisfactory substitutes for measuring CTLs. Thus, the proliferation of lymphocytes from two of the patients in response to contact with PSA or in response to peptides representing putative PSA epitopes suggests an appropriate cellular immune response. The ability of PSA or PSA derived peptides to stimulate cytokine production -- i.e., gamma interferon and IL-4 production -- from lymphocytes in these patients indicates that the cellular response is obtained specifically with respect to PSA. The observation of the development of a positive skin test response to PSA in one patient is also consistent with these observations showing the development of cell mediated immunity in this patient.

5. In my opinion, the results obtained in this clinical study provide evidence that the vaccines are likely to be effective in exerting a beneficial effect on patients with prostate tumors or at risk for prostate tumors, though much further work will be required to increase the frequency and potency of the responses..

6. The efficacy shown for the vaccine tested in the foregoing clinical studies further provides evidence that analogous vaccines based on host tissue antigen, such as prostate specific membrane antigen (PSMA) and prostate acid phosphatase (PAP) would behave in a similar

manner. It is also well known that if the entire antigen is effective as a vaccine, portions of the antigen will be effective as well, especially if manipulated by art-known methods to enhance their immunogenicity, such as by coupling them to carrier.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

October 14, 1996 Philip O. Livingston, MD

CURRICULUM VITAE

NAME: Philip O. Livingston, M.D.

DATE OF BIRTH: January 14, 1943

PLACE OF BIRTH: New York, N.Y.

NATIONALITY: U.S.A.

EDUCATION:

Princeton University, Princeton, New Jersey
B.A. Biology - 1965

Harvard Medical School, Boston, Massachusetts
M.D. - 1969

HOSPITAL APPOINTMENTS:

Cornell Cooperating Hospitals, New York
Intern 7/1/69 - 6/30/70

Cornell Cooperating Hospitals, New York
Resident 7/1/70 - 6/30/71

New York University Hospital, New York
Fellow - Immunology, 7/1/71 - 6/30/73

U.S. Naval Hospital at Roosevelt Road, Puerto Rico
Lt. Cmdr., Chief of Allergy and Rheumatology Services
1973 - 1975

Memorial Hospital, New York - Fellow -
Department of Medicine - Clinical Immunology Service
7/1/75 - 6/30/77

Memorial Hospital, New York - Assistant Attending
Physician - Department of Medicine
Clinical Immunology Service
7/1/77 - 7/25/90

Memorial Hospital, New York - Associate Attending
Physician - Department of Medicine
Clinical Immunology Service
7/26/90 - Present

RESEARCH APPOINTMENTS: Memorial Sloan-Kettering Cancer Center, New York

Research Fellow	7/1/75 - 6/30/77
Research Associate	7/1/77 - 1/1/85
Assistant Member	1/2/85 - 7/25/90
Associate Member	7/26/90 - Present

TEACHING APPOINTMENTS:

Cornell University Medical College
Assistant Professor of Medicine
7/1/77 - 1985

Associate Professor 7/1/93 - Present

Exhibit A

HONORS AND FELLOWSHIPS: American Cancer Society Junior Faculty Clinical Fellow, July 1978 - 1981

BOARD CERTIFICATIONS: American Board of Internal Medicine 1972, recertified 1981: Subspecialty board in Allergy and Immunology, 1973 and Medical Oncology, 1982.

HOME ADDRESS: 156 East 79th Street
New York, N.Y. 10021
(212) 472-3390

OFFICE ADDRESS: 1275 York Avenue
New York, N.Y. 10021
(212) 639-7425

SOCIAL SECURITY: 046-34-7719

MARITAL STATUS: Married SPOUSE'S NAME: Lucy Hann, M.D.

DEPENDENTS: 1

MILITARY STATUS: Lt. Cmdr. U.S. Navy 1973-1975

PUBLICATIONS:

Livingston, P.O., Shiku, M., Bean, M.A., Pinsky, C.M., Oettgen, H.F. and Old, L.J.: Cell-mediated cytotoxicity for cultured autologous melanoma cells. <u>Int. J. Cancer</u> 24: 34-44, 1979.

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Heiling, F., Lloyd, K.O., Oettgen, H.F. and Livingston, P.O. Increased immunogenicity of GD3 ganglioside after covalent attachment to proteins. Proc. Amer. Assoc. Cancer Res. 33: 335, 1992.

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PATENT
Docket No. 204372000320

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being facsimile transmitted to the United States Patent and Trademark Office on November 4, 1996

Nancy J. Robins

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF JEAN CLAUDE BYSTRYN, MD PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Jean Claude Bystryn, MD, declare as follows:

1. I am a member of the Department of Dermatology at the New York University Medical Center. A copy of my *Curriculum Vitae* is attached hereto as Exhibit A. I have no association or connection with Jenner Technologies, the assignee herein.

2. I have reviewed the Declaration Under 37 C.F.R. 1.132 prepared by Dr. Lynn E. Spitler describing the results of a clinical study directed to the use of prostate specific antigen (PSA) as an active ingredient in an antiprostate cancer vaccine. I am also familiar with the study itself, and with the results that were obtained.

3. The purpose of the study was to obtain evidence that the vaccines would raise a sufficient cellular immune response to have a beneficial effect with respect to prostate tumors. Such a result could be shown directly by measuring cytotoxic lymphocyte (CTL) generation; however, I am aware that this was not possible in these studies because the assay was not satisfactory because of the lack of an appropriate target cell for the assay.

4. The responses measured are understood in the art to be satisfactory substitutes for measuring CTLs. Thus, the proliferation of lymphocytes from two of the patients in response to contact with PSA or in response to peptides representing putative PSA epitopes is indicative of an appropriate cellular immune response. The ability of PSA or PSA derived peptides to stimulate cytokine production -- i.e., gamma interferon and IL-4 production -- from lymphocytes in these patients also indicates that the cellular response is obtained specifically with respect to PSA. The observation of the development of a positive skin test response to PSA in one patient is also consistent with these observations showing the development of cell-mediated immunity in the patients.

5. In my opinion, the results obtained in this clinical study provide evidence that the vaccines are likely to be effective in exerting a beneficial effect on patients with prostate tumors or at risk for prostate tumors.

6. The efficacy shown for the vaccine tested in the foregoing clinical studies further provides evidence that analogous vaccines based on host tissue antigen, such as prostate specific membrane antigen (PSMA) and prostate acid phosphatase (PAP) would behave in a similar manner. It is also known that if the entire antigen is effective as a vaccine, portions of the antigen may be effective as well, especially if manipulated by art-known methods to enhance their immunogenicity, such as by coupling them to carrier.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

October 10, 1996

Jean Claude Bystryn, MD

10/30/96

CURRICULUM VITAE

JEAN-CLAUDE BYSTRYN, M.D.

DATE OF BIRTH: May 8, 1938 **PLACE OF BIRTH:** Paris, France (U.S. Citizen)

EDUCATION:

1958 B.S. University of Chicago (biochemistry), Special honors in the natural sciences
1962 M.D. New York University School of Medicine, New York, NY

POST-GRADUATE TRAINING:

1962-1963 Intern (mixed), Montefiore Hospital, New York, NY
1963-1964 Residency (medicine), Montefiore Hospital, New York, NY
1966-1969 Residency (dermatology), NYU School of Medicine, New York, NY
1968-1969 USPH post-doctoral research fellow (vascular physiology), Laboratory of Dr. Chester Hyman, Dept. of Physiology and Dermatology, University of Southern California, Los Angeles, CA
1969-1972 USPH post-doctoral research fellow (immunology), Laboratory of Dr. Jonathan Uhr, Dept. of Medicine, NYU School of Medicine, New York, NY

MEDICAL LICENSURE:

1963 State of New York
1964 State of California

BOARD CERTIFICATION:

1970 American Board of Dermatology
1986 American Board of Immunodermatopathology

MILITARY EXPERIENCE:

1964-1966 USPHS Heart Disease Control Officer, Albany, NY

HOSPITAL AND TEACHING APPOINTMENTS:

1969- Assistant Visiting Dermatologist, Bellevue Hospital Center, New York
1969- Assistant Attending, University Hospital, NYU School of Medicine, New York
1970-1971 Instructor, Dept. of Dermatology, NYU School of Medicine, New York
1970-1997 Associate Attending, Depts. of Dermatology and Syphilology, Bellevue Hospital Center, New York
1971-1972 Assistant Professor of Clinical Dermatology, Dept. of Dermatology, NYU School of Medicine, New York
1972-1976 Assistant Professor, Dept. of Dermatology, NYU School of Medicine, New York
1972- Director, Immunofluorescence Laboratory, Dept. of Dermatology, NYU School of Medicine, New York
1974- Co-Director, Bullous Disease Clinic, Skin and Cancer Unit, NYU School of Medicine, New York
1976-1984 Associate Professor, Dept. of Dermatology, NYU School of Medicine, New York
1982- Director, Melanoma Immunotherapy Clinic, Skin and Cancer Unit, NYU Medical Center, New York
1983-1992 Executive Committee, Kaplan Cancer Center, NYU Medical Center, New York
1983- Director, Melanoma Program, Kaplan Cancer Center, NYU Medical Center, New York
1984- Professor, Dept. of Dermatology, NYU School of Medicine, New York

Exhibit A

HONORS AND AWARDS:

1954	Ford Foundation Fellowship for early entrance to colleg
1968	Husik Prize in Dermatology, NYU School of Medicin
1979	Irma T. Hirschl Career Scientist Award
1985	Skin Cancer Foundation Annual Award
1987	Philippine Society of Dermatology, Honorary Member
1990	AOA
1990	Society Francaise de Dermatologie, Honorary Member
1991	The Hellenic Society of Dermatology, Honorary Member

CHAIRMAN OF SYMPOSIA/WORKSHOPS AT NATIONAL AND INTERNATIONAL CONFERENCES:

1982	Int'l Symposium on Stratum Corneum, Cardiff
1982	Fifth Southeast Asian Conference on Dermatology, Manila
1982	XVIth Int'l Congress on Dermatology, Tokyo
1983	Joint Annual Meeting of the Society of Investigative Dermatology and European Society for Dermatologic Research, Washington, DC
1983	First World Congress on Cancers of the Skin, New York
1983	XIIth Int'l Pigment Cell Conference
1985	Second World Congress on Cancer of the Skin, New York
1985	Joint International Meeting of the SID and JCID, Washington, DC
1987	XVIIth International Congress of Dermatology, Berlin
1988	Second International Conference on Melanoma, Venice
1990	NIH Research Workshop on Alopecia Areata, Organizer
1992	XVIIIth International Congress of Dermatology, New York
1993	Third International Conference on Melanoma, Venice
1993	New York Academy of Sciences Conference on "Specific Immunotherapy of Cancer with Vaccines," Washington, D.C., Conference Chair
1994	International Symposium "Skin Therapy Update '94," Creta, Co-Chairman
1995	Sixth World Congress of Cancers of the Skin, Buenos Aires, Chairperson of Plenary Session on Basic Science
1996	International Conference for Apheresis 1996, Kyoto, Chair

PROFESSIONAL SOCIETIES:

Society Francaise de Dermatologie, Honorary Member
Philippine Society of Dermatology, Honorary Member
American Academy of Dermatology
American Dermatological Association
American Association of Immunologists
American Association for Cancer Research
Society for Investigative Dermatology
American Society for Cell Biology
International Society for Tropical Dermatology
American Federation for Clinical Research
New York Dermatological Society
Dermatology Foundation
International Pigment Cell Society
PANAMERICAN Society for Pigment Cell Research
International Society for Vaccines
Clinical Immunology Society
Society for Biological Therapy

ASSIGNMENTS IN PROFESSIONAL SOCIETIES OR MEDICAL JOURNALS:

1975-1978	Mycosis Fungoides Cooperative Group, Chairman, Immunotherapy Panel
1980-1982	Dystrophic Epidermolysic Bullosa Foundation, Board of Medical Advisors
1980-	Skin Cancer Foundation, Board of Medical Advisors and Chairman Grant Review Committee
1981-1990	American Academy of Dermatology, Task Force on Immunopathology
1986-1987	New York Dermatological Society, Secretary
1987-1988	New York Dermatological Society, President
1983-	National Alopecia Areata Foundation, Medical Advisory Board
1987-	Molecular Therapeutic, Editorial Board
1990-	Vaccine Research, Editorial Board
1992-1996	Journal of the European Academy of Dermatology and Venereology, Editorial Board
1993-	Society for Investigative Dermatology, Committee for Industrial Sponsorship
1993-	American Dermatological Association, Membership Committee
1993-	National Vitiligo Foundation, Medical Advisory Board
1994-	American Academy of Dermatology, Commission on Melanoma
1994-	American Board of Dermatology, Training Program in Clinical and Laboratory Dermatological Immunology Review Committee
1994-	American Board of Dermatology, Recertification Committee
1995-	American Skin Association, Medical/Scientific/Policy Advisory Committee
1996	NIH Ad Hoc Reviewer, Biological Response Modifier Program
1996-2000	American Academy of Dermatology, Manpower Committee

PUBLICATIONS

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2. **BYSTRYN J-C**, FREEDMAN RI, HYMAN C. Clearance of iodoantipyrene from Mecholyl blanched skin in atopics. Arch Derm 100:165, 1969.

3. LEVAN NE, **BYSTRYN J-C**, HYMAN C. Temperature and blood flow in macules of lepromatous l prosy. Int J Leprosy 37:249, 1969.

4. **BYSTRYN J-C**, GRAF MW, UHR JW. Regulation of antibody formation by serum antibody. II. Removal of specific antibody by means of exchange transfusion. J Exp Med 132:1279, 1970.

5. UHR JW, **BYSTRYN J-C**, GRAF MW. The regulation of antibody formation. In: Morphological and Fundamental Aspects of Immunity, ed. by Lindahl-Kiessling, Alm and Hanna; Plennum Press, New York, 1971, pp 395.

6. **BYSTRYN J-C**, FELLNER M. Erythema multiforme. In: Immunological Diseases, 2nd edition, ed. by Samter; Little, Brown and Co., Boston, MA, 1971.

7. SCHENKEIN I, **BYSTRYN J-C**, UHR JW. Specific removal of in vivo antibody by extracorporeal circulation of an immunoabsorbent in gel. J Clin Invest 50:1864, 1971.

8. **BYSTRYN J-C**, SCHENKEIN I, UHR JW. A model for the regulation of antibody synthesis by serum antibody. In: Progress in Immunology, vol. 1, ed. by B Amos; Academic Press, New York, 1971, pp 627.

9. **BYSTRYN J-C**. Drug fever. Am J Med Science 264:467, 1972.

10. **BYSTRYN J-C**, BART RS, LIVINGSTON P, KOPF AW. Immunity to murine melanoma. J Invest Derm 60:249, 1973.

11. **BYSTRYN J-C**, SISKIND GW, UHR JW. Binding of antigen by immunocytes 1. Effect of ligand valency on binding affinity of MOPC 315 cells for DNP-conjugates. J Exp Med 137:301, 1973.

12. BYSTRYN J-C, ABEL E, WEIDMAN A. Antibodies against the cytoplasm of human epidermal cells. Arch Derm 108:241-244, 1973.

13. UHR JW, SCHENKEIN I, BYSTRYN J-C. Immunoregulation. In: Specific Receptors of Antibodies, Antigens, and Cells. (Third Int'l Convocation f Immunology, Buffal , NY) S. Karger, Basel, 1973, pp 397.

14. BYSTRYN J-C, SCHENKEIN I, UHR JW. Double antibody radioimmunoassay for tumor antibodies. J Natl Cancer Inst 52:911-915, 1974.

15. BYSTRYN J-C, SCHENKEIN I, BAUR S, UHR JW. Partial isolation and characterization of antigen(s) associated with murine melanoma. J Natl Cancer Inst 52:1263-1269, 1974.

16. BYSTRYN J-C, BART RS, LIVINGSTON P, KOPF AW. Growth and immunogenicity of B16 murine melanoma. J Invest Derm 63:369-373, 1974.

17. BYSTRYN J-C, ABEL E, DEFEO C. Pemphigus foliaceus: subcorneal intercellular antibodies of uniqu specificity. Arch Derm 110:857-862, 1974.

18. ROBINS DN, DEFEO C, BYSTRYN J-C. Hepatotoxicity of azathioprine. Cutis 14:600, 1974.

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20. BYSTRYN J-C. Nature of antigens associated with murine melanoma. In: Pigment Cell, ed. by V Riley, S Karger, Basel, 2:158, 1976.

21. ABEL EA, BYSTRYN J-C. Epidermal cytoplasmic antibodies: incidence and type in normal persons and patients with melanoma. J Invest Derm 66:44-48, 1976.

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25. BYSTRYN J-C, RODRIGUEZ J. Pemphigus foliaceus associated with absence of intercellular antigens in th lower layers of the epidermis. Arch Derm 113:1696, 1976.

26. BYSTRYN J-C. Clinical significance of basal cell layer antibodies. Arch Derm 113:1380-1382, 1977.

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34. AUERBACK R, **BYSTRYN J-C**. Effect of plasmapheresis and immunosuppressive therapy of intercellular antibodies in pemphigus vulgaris. Arch Derm 115:728-730, 1979.

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I hereby certify that this correspondence is being facsimile transmitted to the United States Patent and Trademark Office on November 4, 1996

Nancy J. Robins

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF MICHAEL MASTRANGELO, MD PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Michael Mastrangelo, MD, declare as follows:

1. I am a Professor of Medicine, and Associate Clinical Director of the Jefferson Cancer Center in Philadelphia, Pennsylvania. A copy of my *Curriculum Vitae* is attached hereto as Exhibit A. I am Chairman of the Scientific Advisory Board of Jenner Technologies, the assignee in this application, and a shareholder in the company.

2. I have reviewed the Declaration Under 37 C.F.R. 1.132 prepared by Dr. Lynn E. Spitler describing the results of a clinical study directed to the use of prostate specific antigen (PSA) as an active ingredient in an antiprostate cancer vaccine. I am also familiar with the study itself, and with the results that were obtained.

3. The purpose of the study was to obtain evidence that the vaccines would raise a sufficient cellular immune response to have a beneficial effect with respect to prostate tumors. Such a result could be shown directly by measuring cytotoxic lymphocyte (CTL) generation; however, I am aware that this was not possible in these studies because the assay was not satisfactory because of the lack of an appropriate target cell for the assay.

4. The responses measured are understood in the art to be satisfactory substitutes for measuring CTLs. Thus, the proliferation of lymphocytes from two of the patients in response to contact with PSA or in response to peptides representing putative PSA epitopes is indicative of an appropriate cellular immune response. The ability of PSA or PSA derived peptides to stimulate cytokine production -- i.e., gamma interferon and IL-4 production -- from lymphocytes in these patients also indicates that the cellular response is obtained specifically with respect to PSA. The observation of the development of a positive skin test response to PSA in one patient is also consistent with these observations showing the development of cell-mediated immunity in the patients.

5. In my opinion, the results obtained in this clinical study provide evidence that the vaccines are likely to be effective in exerting a beneficial effect on patients with prostate tumors or at risk for prostate tumors.

6. The efficacy shown for the vaccine tested in the foregoing clinical studies further provides evidence that analogous vaccines based on host tissue antigen, such as prostate specific membrane antigen (PSMA) and prostate acid phosphatase (PAP) would behave in a similar manner. It is also well known that if the entire antigen is effective as a vaccine, portions of the antigen will be effective as well, especially if manipulated by art-known methods to enhance their immunogenicity, such as by coupling them to carrier.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

October 10, 1996 Michael Mastrangelo, MD

Revised 12/93

CURRICULUM VITAE

NAME: Michael J. Mastrangelo, M.D. SOCIAL SECURITY NO: 210-28-9391

DATE OF BIRTH: October 3, 1938

MARITAL STATUS: Married 1964 - Ann Sunday
Children: David M. 1965
Mark S. 1967
Audrey A. 1970

EDUCATION

Villanova University, Villanova, PA
B.S. (Biology), Summa Cum Laude (1/696) 1956-1960
The Johns Hopkins University School of Medicine, M.D. 1960-1964

POSTGRADUATE TRAINING

Thomas Jefferson University Hospital, Philadelphia, PA
Medical Intern 1964-1965
Medical Resident 1967-1969
American Cancer Society Trainee 1968-1969
Oncology Fellow 1969-1970
Chief Medical Resident 1970-1971
Post-doctoral Fellow 1971-1972
Laboratory of R. T. Prehn, M.D.
The Institute for Cancer Research
7701 Burholme Avenue
Philadelphia, PA 19111

MILITARY SERVICE

CPT, MC, U.S. Army, Molecular Biology Branch,
Medical Laboratories, Edgewood Arsenal, MD 1965-1967

SPECIALTY CERTIFICATION

Diplomate of the American Board of Internal Medicine 1972
Certificate #37358
Diplomate, subspecialty of Medical Oncology 1973
Certificate #37358

LICENSURE

Maryland - D7736 1964
Pennsylvania - MD 008521-E 1965
New Jersey - MA24857 1971

HONORARY SOCIETIES

Delta Epsilon Sigma 1960
National Collegiate Who's Who 1960
Sigmi Xi 1984

Exhibit A

PROFESSIONAL SOCIETIES

College of Physicians of Philadelphia	197[illegible]
Philadelphia County Medical Society	1972-1986
Pennsylvania Medical Society	1972-1986
American Medical Association	1972
American Federation for Clinical Research	1973
American Association for the Advancement of Science	1973
American Society of Clinical Oncology	1973
American College of Physicians - Member	1975
- Fellow	1974
American College of Clinical Pharmacology Fellow	1974
American Association for Cancer Research	1979-1980
Program Committee for Immunology - Member	1987-1988
" " " " - Chairperson	1984-1985
Membership Committee	1993-1994
Gertrude Elion Award Committee	1981-
Society for Biological Therapy	1988-1991
Board of Directors	1989-1990
Chairman, Program and Publications Committee	1990-1992
President	

PROFESSIONAL ACTIVITIES

Seminars in Oncology - Associate Editor	1974-
Eastern Cooperative Oncology Group - Member	1973-1976
Malignant Melanoma Clinical Cooperative Group - Member	1973-1977
Committee on Tumor Immunotherapy, DCBD, NCI - Member	1975-1979
American Cancer Society, Philadelphia Division	
Member, Professional Education Committee	1975-1977
Member, Board, Northeast Unit	1978-1979
American Cancer Society, National Office	
Member, Advisory Committee on Immunology and Immunotherapy	1987-1991
Philadelphia County Medical Society -	1976-1984
Member, Cancer Control Subcommittee	1976-1977
Experimental Therapeutic Study Section, NIH -	
Member	1980-1982
Chairman I + II	1982-1984
Cancer Immunology and Immunotherapy - Board of Editors	1981-1993
Subcommittee on Biological Response Modifiers of the	1978-1980
Division of Cancer Treatment's Board of Scientific Counsellors	
Biological Response Modifier Program, DCT, NCI -	
Operating Committee, Member	1980-1982
Decision Network Committee, Member	1980-1984
Journal of Biological Response Modifiers - Editoral Board	1982-1990
Journal of Immunotherapy - Editorial Board	1990-
Cancer Research - Associate Editor	1983-1994
Hybridoma - Editorial Board	1987-1992
PDQ - Extramural B ard	1989-1993
The United States Pharmacopeial Conv ntion, Inc.	
Advis ry Panel on Hemat l gic and Neoplastic Diseases	1990-1995
Vaccine Research - Associate Edit r, Tum r Vaccines	1990-

FACULTY AND APPOINTMENTS

Current -

Professor of Medicine,	1984-
Director, Division Medical Oncology	1984-1993
Member, Division Neoplastic Diseases	1993-
Jefferson Medical College	
1025 Walnut Street	
Philadelphia, PA 19107	
Courtesy Staff	1987-
Associate Staff	1984-1987
Associate Physician (Medicine)	1977-1984
Assistant Physician (Medicine)	1972-1976
Director, Pigmented Lesion Clinic	1972-1984
American Oncologic Hospital	
Central and Shelmire Avenues	
Philadelphia, PA	
Consultant, Oncology	1973-
Department of Medicine	
The Mercer Medical Center	
Trenton, NJ	
Consultant, Oncology Division	1974-
Retina Service	
Wills Eyes Hospital	
Philadelphia, PA	

Prior

Instructor (Medicine)	1970-1974
Assistant Physician (Medicine)	1971-1974
Jefferson Medical College and Hospital	
Philadelphia, PA 19107	
Clinical Associate Professor (Medicine)	1977-1984
Clinical Assistant Professor (Medicine)	1974-1977
School of Medicine	
Temple University Health Science Center	
Philadelphia, PA 19107	
Research Physician	1972-1980
The Institute for Cancer Research	
Philadelphia, PA	
Consultant, Medical Oncology	1976-1986
Doylestown Hospital	
Doylestown, PA	
Consultant, Tumor Immunotherapy	1973-1988
Chestnut Hill Hospital	
Philadelphia, PA	

PUBLICATIONS

APERS

1. Mastrangelo, M.J., Giordano, W.P. and Johnson, R.P. Surface behavior of individual lipids similar to constituents of pulmonary surfactant. EATR 4087, April 1967.

2. Mastrangelo, M.J. and Johnson, R.P. Segmental reversibility and hysteresis of preparations similar to pulmonary surfactant. EATR 4097, May 1967.

3. Mastrangelo, M.J., Carwile, H. and Johnson, R.P. Qualitative protein composition of lung surfactant preparations. EATR 4124, August 1967.

*4. Weiss, A.J. and Mastrangelo, M.J. Phase I study of a combination of azotomycin (NSC-56654) and 5-Fluorouracil (NSC-19893) in malignant disease. Cancer Chemother Rpt. 54:109-112, 1970.

5. Mastrangelo, M.J. and Weiss, A.J. The chemotherapy of respiratory tract neoplasms. In: Cancer Chemotherapy II, I. Brodsky, S.B. Kahn, J.H. Moyer (Editors), Grune and Stratton, Inc., New York, NY, 1972, pp. 195-208.

*6. Weiss, A.J., Stambaugh, J.E., Mastrangelo, M.J., Laucius, J.F. and Bellet, R.E. A Phase I study of 5-azacytidine (NSC-102816). Cancer Chemother. Rpt. 56:413-420, 1972.

7. Mastrangelo, M.J., Sulit, H.O., Chee, D. and Engstrom, P.F. Cancer Forum: Malignant Melanoma. Penna. Med. 75:43, 1972.

*8. Mastrangelo, M.J., Grage, T. Bellet, R.E. and Weiss, A.J. A Phase I study of emetine hydrochloride (NSC-33669) in solid tumors. Cancer 31:1170-1175, 1973.

*9. Bellet, R.E., Mastrangelo, M.J., Engstrom, P.F. and Custer, R.P. Hepatotoxicity of 5-azacytidine (NSC-102816): A clinical and pathologic study. Neoplasma 20:303-310, 1973.

*10. Bellet, R.E., Mastrangelo, M.J., Dixon, L.M. and Yarbro, J.W. A Phase I study of ICRF 159 (NSC-129943) in solid tumors. Cancer Chemother. Rpt. 57:185-189, 1973.

11. Mastrangelo, M.J., Creech, R.H. and Engstrom, P.F. Cancer Forum: Early diagnosis holds promise in lung cancer. Penna. Med. 76:64, 1973.

12. Bornstein, R.S., Mastrangelo, M.J., Sulit, H.L., Chee, D.O., Yarbro, J.W., Prehn, L.M. and Prehn, R.T. Immunotherapy of melanoma with intralesional BCG. Natl. Cancer Inst. Monogr. 39:213-220, 1973.

*13. Mastrangelo, M.J., Kim, Y.H., Bornstein, R.S., Chee, D.O., Sulit, H.L., Yarbro, J.W. and Prehn, R.T. Clinical and histological correlation of melanoma regression following intralesional BCG therapy: A case report. J. Natl. Cancer Inst. 52:19-24, 1974.

*14. Bellet, R.E., Mastrangel , M.J., Engstrom, P.F., Strawitz, J.G. and Weiss, A.J. Clinical trial with subcutaneously administered 5-Azacytidine (NSC-102816). Cancer Chemother. Rpt. 58:217-222, 1974.

* Reviewed Journal

*15. Bodurtha, A., Kim, Y.H., Laucius, J.F., Donato, R.A. and Mastrangelo, M.J. Hepatic granulomas and other hepatic lesions associated with BCG immunotherapy for cancer. Am. J. Clin. Pathol. 61:747-752, 1974.

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*19. Bodurtha, A.J., Chee, D.O., Laucius, J.F., Mastrangelo, M.J. and Prehn, R.T. Clinical and immunological significance of human melanoma cytotoxic antibody. Cancer Res. 38:189-193, 1975.

*20. Creech, R.H., Catalano, R.B., Mastrangelo, M.J. and Engstrom, P.F. Low dose cyclophosphamide, methotrexate, and 5-fluorouracil therapy of metastatic breast cancer. Cancer 35:1101-1107, 1975.

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*23. Sieper, W.J., Mastrangelo, M.J. and Bellet, R.E. Phase II study of adriamycin (NSC-123127) in patients with metastatic melanoma. Cancer Chemother. Rpt. 59:1181-1182, 1975.

*24. Pliskin, M.E., Mastrangelo, M.J., Brown, A.M. and Custer, R.P. Melanoma of the maxilla presenting as a gingival swelling. Oral Surg. Oral Med. Oral Pathol. 41:101-104, 1976.

*25. Mastrangelo, M.J., Sulit, H.L., Prehn, L.M., Bornstein, R.S., Yarbro, J.W. and Prehn, R.T. Intralesional BCG in the treatment of metastatic malignant melanoma. Cancer 37:684-692, 1976.

*26. Bodurtha, A.J., Berkelhammer, J., Kim, Y.H., Laucius, J.F. and Mastrangelo, M.J. A clinical, histological and immunological study of a case of metastatic malignant melanoma undergoing spontaneous remission. Cancer 37:735-742, 1976.

*27. Ainsworth, A.M., Clark, W.H., Jr., Mastrangelo, M.J. and Conger, K.B. Primary malignant melanoma of the urinary bladder. Cancer 37:1928-1936, 1976.

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28. Bellet, R.E. and Mastrangelo, M.J. Investigational new drugs. In: Oncologic Medicine, P.F. Engstrom, A.I. Sutnick (Editors), University Park Press, Baltimore, MD, 1976, pp. 237-245.

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*32. Bellet, R.E., Mastrangelo, M.J., Laucius, J.F. and Bodurtha, A.J. A randomized prospective trial of DTIC (NSC-45388) alone versus BCNU (NSC-409962) plus vincristine (NSC-67574) in the treatment of metastatic malignant melanoma. Cancer Treat. Rpt. 60:595-600, 1976.

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*34. Bellet, R.E., Catalano, R.B., Danna, V.G., Berkelhammer, J. and Mastrangelo, M.J. A study of antitumor (Phase II) and immunosuppressive effects of ICRF-159 (NSC-129943) in patients with metastatic melanoma. J. Clin. Pharm. 16:433-438, 1976.

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*39. Bellet, R.E., Vaisman, I., Mastrangelo, M.J. and Lustbader, E. Multiple primary malignancies in patients with cutaneous melanoma. Cancer 40:1974-1981, 1977.

* Reviewed Journal

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42. Mastrangelo, M.J., Clark, W.H., Jr., Bellet, R.E. and Berd, D. Cutaneous malignant melanoma: Diagnosis prognosis and conventional therapy. In: Immunotherapy of Cancer: Current Status of Trials in Man, W. Terry, D. Windhorst (Editors), Progress in Cancer Research and Therapy, Vol. 6, Raven Press, New York, NY, 1978, pp. 1-17.

43. Engstrom, P.F., Paul, A.R., Catalano, R.B., Mastrangelo, M.J. and Creech, R.H. Fluorouracil versus fluorouracil + BCG in colorectal adenocarcinoma. In: Immunotherapy of Cancer: Current Status of Trials in Man, W. Terry, D. Windhorst (Editors), Progress in Cancer Research and Therapy, Vol. 6, Raven Press, New York, NY, 1978, pp. 587-596.

*44. Bellet, R.E., Catalano, R.B., Mastrangelo, M.J. and Berd, D. Phase II study of subcutaneously administered 5-azacytidine (NSC-102816) in patients with metastatic malignant melanoma. Med. Pediatr. Oncol. 4:11-15, 1978.

45. Greene, M.H., Reimer, R.R., Clark, W.H., Jr. and Mastrangelo, M.J. Precursor lesions in familial melanoma. Semin. Oncol. 5:85-87, 1978.

*46. Bellet, R.E., Catalano, R.B., Mastrangelo, M.J., Berd, D. and Koons, L.S. Phase II trial of VM-26 (NSC-122819) in patients with metastatic melanoma. Cancer Treat. Rpt. 62:445-447, 1978.

*47. Clark, W.H., Jr., Reimer, R.R., Green, M., Ainsworth, A.M. and Mastrangelo, M.J. Origin of familial malignant melanoma from heritable melanocytic lesions. The B-K mole syndrome. Arch. Derm. 114:732-738, 1978.

48. Berkelhammer, J., Mastrangelo, M.J., Bellet, R.E., Prehn, R.T. and Thibault, L.H. Failure of lymphocyte microcytoxicity to distinguish relapsers from non-relaps in melanoma patients receiving postsurgical adjuvant chemotherapy. Eur. J. Cancer 14:793-798, 1978.

49. Mastrangelo, M.J., Berd, D. and Bellet, R.E. Limitations, obstacles and controversi in the optimal development of immunotherapy. In: Immunotherapy of Human Cancer, E. Hersh, J. Sinkovics (Editors), Raven Press, New York, NY, 1978, pp. 375-394.

50. Bellet, R.E., Mastrangelo, M.J., Berd, D. and Lustbader, E. Melanoma chemotherapy. In: Cancer Chem therapy III, I Brodsky, S.B. Kahn, J.F. Conroy (Editors), Grune and Stratt n, Inc., New York, 1978, pp. 225-242.

51. Mastrangelo, M.J. and Bellet, R.E. Immunotherapy of melanoma. In: Clinical Therapeutics, D.T. Lowenthal, D.A. Major II, (Edit rs), Grune and Stratton, Inc., New York, 1978, pp. 281-291.

* Reviewed J urnal

*52. Bellet, R.E., Catalano, R.B., Mastrangelo, M.J. and Berd, D. A positive Phase II trial of dibromodulcitol in patients with metastatic melanoma refractory to DTIC and nitrosourea. Cancer Treat. Rpt. 62:2095-2099, 1978.

53. Bellet, R.E., Mastrangelo, M.J. and Berd, D. Chemotherapy of metastatic malignant melanoma. In: Human Malignant Melanoma, Grune and Stratton, Inc., New York, 1979, pp. 355-416.

54. Vaisman, I., Mastrangelo, M.J., Bellet, R.E. and Lustbader, E. Primary cutaneous melanoma and coexisting malignant neoplasms. In: Human Malignant Melanoma, Grune and Stratton, Inc., New York, 1979, pp. 243-260.

55. Mastrangelo, M.J., Bellet, R.E. and Berd, D. Immunology and immunotherapy of malignant melanoma. In: Human Malignant Melanoma, Grune and Stratton, Inc., New York, 1979, pp. 355-416.

56. Mastrangelo, M.J., Bellet, R.E. and Berd, D. Adjuvant therapy. In: Human Malignant Melanoma, Grune and Stratton, Inc., New York, 1979, pp. 309-324.

57. Mastrangelo, M.J., Bellet, R.E. and Berd, D. Prognostic Factors. In: Human Malignant Melanoma, Grune and Stratton, Inc., New York, 1979, pp. 273-281.

58. Berd, D., Mastrangelo, M.J. and Bellet, R.E. Metastatic uveal melanoma. In: Human Malignant Melanoma, Grune and Stratton, Inc., New York, 1979, pp. 489-496.

59. Laucius, J.F. and Mastrangelo, M.J. Cutaneous depigmentary phenomena in patients with malignant melanoma. In: Human Malignant Melanoma, Grune and Stratton, Inc., New York, 1979, pp. 209-226.

*60. Berkelhammer, J., Mastrangelo, M.J., Bellet, R.E., Berd, D. and Prehn, R.T. Chemoimmunotherapy increases the lymphocyte reactivity of melanoma patients. Eur. J. Cancer 15:197-204, 1979.

*61. Mastrangelo, M.J., Bellet, R.E. and Berd, D. A Phase III comparison of methyl-CCNU + vincristine with or without BCG + allogeneic tumor cells in metastatic melanoma. Cancer Immunol. Immunother. 6:231-236, 1979.

*62. Goepp, C.E., Mastrangelo, M.J., Patel, Y.A. and Clark, W.H., Jr. Cutaneous malignant melanoma in a patient with neurofibromatosis. Arch. Dermatol. 115:864-1979.

*63. Berd, D., Wilson, E.J., Bellet, R.E. and Mastrangelo, M.J. The effect of methyl-C adjuvant therapy on immune responses of patients with malignant melanoma. Cancer Res. 39:4472-4476, 1979.

*64. Bellet, R.E., Danna, V., Mastrangelo, M.J. and Berd, D. Evaluation of a "nude" mouse-human tumor panel as a predictive secondary screen for cancer chemotherapeu agents. J. Natl. Cancer Inst. 63:1185-1188, 1979.

65. Tems-Reines, S.J. and Mastrangelo, M.J. In: Clinical Immunotheapy, A. F. LoBuglio (Edit r), Marcel Dekker, Inc., NY, 1980, pp. 7-29.

* Reviewed Journal

*66. Augsburger, J.J., Shields, J.A., Mastrangelo, M.J. and Frank, P.F. Diffuse primary malignant melanoma of the choroid arising in a patient with prior cutaneous malignant melanoma. Arch. Ophthalmol. 98:1261-1264, 1980.

*67. Herlyn, M., Clark, W.H., Jr., Mastrangelo, M.J., Guerry, D. IV, Elder, D.E., LaRossa, D., Hamilton, R., Bondi, E.M., Tuthill, R., Steplewski, Z. and Koprowski, H. Specific immunoreactivity of monoclonal anti-melanoma antibodies. Cancer Res. 40:3602-3609, 1980.

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72. Mastrangelo, M.J., Rosenberg, S.A., Baker, A. and Katz, H. Cutaneous melanoma. In: Cancer Principles and Practices of Oncology, V.T. DeVita, S. Hellerman, S. Rosenberg (Editors), J.B. Lippincott, Co., Philadelphia, PA, 1982, pp. 1124-1138

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*77. Donos , L.A., Nagy, R.M., Brockman, R.J., Augsburger, J.J., Shields, J.A., Berd, D. and Mastrangel , M.J. Metastatic uveal melanoma. Hepatic cell-surface enzymes, isoenzymes and serum sialic acid levels in early metastatic disease. Arch. Ophthalmol. 101:791-794, 1983.

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*78. Berd, D., Maguire, H.C. and Mastrangelo, M.J. Impairment of concanavalin A-inducible suppressor activity following administration of cyclophosphamide to patients with advanced cancer. Cancer Res. 44:1275-1280, 1984.

*79. Mastrangelo, M.J., Berd, D. and Maguire, H.C. The current condition and prognosis of tumor immunotherapy: A second opionion. Cancer Treat. Rpt. 68:207-21[illegible], 1984.

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PATENT
Docket No. 204372000320

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence is being facsimile transmitted to the United States Patent and Trademark Office on November 4, 1996.

Nancy J. Robins

Nancy J. Robins

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF ROBERT OLDHAM, MD PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Robert Oldham, MD, declare as follows:

1. I am the Director of the Biological Therapy Institute in Franklin, Tennessee. A copy of my *Curriculum Vitae* is attached hereto as Exhibit A. I am a consultant to Jenner Technologies, the assignee of the present application.

2. I have reviewed the Declaration Under 37 C.F.R. 1.132 prepared by Dr. Lynn E. Spitler describing the results of a clinical study directed to the use of prostate specific antigen (PSA) as an active ingredient in an antiprostate cancer vaccine. I am also familiar with the study itself, and with the results that were obtained.

3. The purpose of the study was to obtain evidence that the vaccines would raise a sufficient cellular immune response to have a beneficial effect with respect to prostate tumors. Such a result could be shown directly by measuring cytotoxic lymphocyte (CTL) generation; however, I am aware that this was not possible in these studies because the assay was not satisfactory because of the lack of an appropriate target cell for the assay.

4. The responses measured are understood in the art to be satisfactory substitutes for measuring CTLs. Thus, the proliferation of lymphocytes from two of the patients in response to contact with PSA or in response to peptides representing putative PSA epitopes is indicative of an appropriate cellular immune response. The ability of PSA or PSA derived peptides to stimulate cytokine production -- i.e., gamma interferon and IL-4 production -- from lymphocytes in these patients also indicates that the cellular response is obtained specifically with respect to PSA. The observation of the development of a positive skin test response to PSA in one patient is also consistent with these observations showing the development of cell-mediated immunity in the patients.

5. In my opinion, the results obtained in this clinical study provide evidence that the vaccines are likely to be effective in exerting a beneficial effect on patients with prostate tumors or at risk for prostate tumors.

6. The efficacy shown for the vaccine tested in the foregoing clinical studies further provides evidence that analogous vaccines based on host tissue antigen, such as prostate specific membrane antigen (PSMA) and prostate acid phosphatase (PAP) would behave in a similar manner. It is also well known that if the entire antigen is effective as a vaccine, portions of the antigen will be effective as well, especially if manipulated by art-known methods to enhance their immunogenicity, such as by coupling them to carrier.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

October 10, 1996 Robert Oldham, MD

Revised: 10/1/95

CURRICULUM VITA

Name.	Robert K. Oldham, M.D.
Permanent Address	357 Riverside Drive Franklin, Tennessee 37064 615/791-4073
Social Security #:	500-40-8139
Date/Place of Birth:	9/16/41; Pocatello, Idaho
Marital Status:	Single (5 sons)
College:	University of Missouri, Columbia, MO Chemical Engineering Pre-Medical Sciences
Medical School:	1968, Medical Degree University of Missouri Columbia, Missouri
Graduate Training:	1970-1971 Immunology NIH Graduate Program

Professional Employment

1991 - Present	Chairman of the Board and President Cancer Therapeutics Inc. 357 Riverside Drive Franklin, Tennessee 37064
1992 - Present	Chairman of the Board and President American Patient Services 357 Riverside Drive Franklin, Tennessee 37064
1986 - Present	Chairman f the Board Media America 357 Riversid Drive Franklin, Tennessee 37064

Consultant:

1984 - 1986	Wellcome Biotechnology Ltd. Kent, England
1989 - Present	Amersham International Buckinghamshire, England
1991 - Present	Cancer Treatment Centers of America Zion, Illinois
1994 - Present	Applied Immune Sciences Santa Clara, California 95054

Editorial Boards:

1992 - Present	Cancer Biotherapy, Founder, (now Cancer Biotherapy & Radiopharmaceuticals) Editor-in-Chief
1990 - Present	In Vivo, Editorial Board Natural Immunity and Cell Growth Regulation, Editorial Board
1988 - 1992	Molecular Biotherapy, Founder and Editor-in-Chief
1982 - 1989	Journal of Biological Response Modifiers (now Journal of Immunotherapy) Founder and Editor-in-Chief
1982 - 1985	Cancer Immunology and Immunotherapy, Editorial Board

Major Research Interests:

Cancer Biotherapy
Biologicals and Biological Response Modifiers
Investigative Trials in Clinical Oncology
Cellular Therapy of Cancer
Monoclonal Antibodies & Immunoconjugates in Cancer Treatment
In Vitro Assays in Tumor Immunology
Privatization of Cancer Research

Professional Employment (Continued)

1984 - Present	Founder and Director Biological Therapy Institute 357 Riverside Drive Franklin, Tennessee 37064
1984 - Present	Clinical Professor of Medicine Hematology/Oncology University of Missouri Columbia, Missouri 65212
1984 - 1990	Founder, Scientific Director and Chairman of the Board Biotherapeutics Inc. (Now Response Technologies, Inc. Technologies, Inc.) Memphis, Tennessee 38117
1984 - 1986	Founder and Consultant Biomedical Research Center University of British Columbia Vancouver, British Columbia V6T 2B5
1980 - 1984	Founding Director, Biological Response Modifiers Program Associate Director, Division of Cancer Treatment National Cancer Institute Frederick, Maryland 21701
1975 - 1980	Founder and Director, Div. of Oncology Associate Professor of Medicine Associate Director; Cancer Center Vanderbilt University Medical Center Nashville, Tennessee 37232

Professional Employment Continued:

1974 - 1975	Senior Investigator Laboratory of Immunodiagnosis National Cancer Institute Bethesda, Maryland 20205
1973 - 1974	Senior Investigator Cellular and Tumor Immunology Section Laboratory of Cell Biology National Cancer Institute Bethesda, Maryland 20205
1972 - 1973	Research Associate with Professor G. Mathe ICIG - Hospital Paul Brousse Villejuif, France
1971 - 1972	Clinical Associate, Cellular and Tumor Immunology Section Laboratory of Cell Biology National Cancer Institute Bethesda, Maryland 20205
1970 - 1971	Clinical Associate, Radiation Branch National Cancer Institute Bethesda, Maryland 20205
1968 - 1970	Internal Medicine Intern and Resident Vanderbilt University Hospital Nashville, Tennessee 37205

Clinical Group Appointments:

1976 - 1980	Principal Investigator - Vanderbilt Southeastern Oncology Study Group (NCI)
1978 - 1980	Principal Investigator - Vanderbilt Lung Cancer Study Group (NCI)
1986 - 1990	Founder and Group Chairman - National Biotherapy Study Group
1986-Present	Principal Investigator - Biological Therapy Institute - National Biotherapy Study Group

Fellowship Experience:

1970 - 1972	Medical Oncology Fellowship, National Cancer Institute - Bethesda, Maryland
1967	PHS Cancer Clinical Fellowship, Lemuel Shattuck Hospital-Jamaica Plain - Boston, Massachusetts
1967	PHS Cancer Clinical Fellowship, Ellis Fischel Cancer Hospital - Columbia, Missouri
1965	PHS Student Research Fellowship, University of Missouri Medical School - Columbia, Missouri

Honors, Awards and Societies:

1967	Alpha Omega Alpha
1970	Diplomat, National Board of Medical Examiners
1975	Diplomat, American Board of Internal Medicine
1975	Diplomat, Medical Oncology, American Board of Internal Medicine
1976	American Association of Immunologists
1976	International Society for Experimental Hematology
1976	Williamson County Medical Society
1977	Southern Medical Association
1977	American Federation for Clinical Research
1977	American Society for Clinical Oncology
1978	American College of Physicians, Fellow
1979	Southern Society for Clinical Investigation
1982	American Osler Society
1984	Society for Biological Therapy, Founder President - May 1984 to November 1986
1984	Reticuloendothelial Society
1986	National Biotherapy Study Group, Founder Group Chairman - 1986 to 1990
1990	Association of Community Cancer Centers
1994	International Cytokine Society
1994	The American Association of Bioethics

Licensure:

Tennessee, Maryland and Missouri

Major Accomplishments (Curriculum Vitae #)

Discovery of Natural Killer Cells
(#8, 9, 27, 28, 31, 46, 47, 56, 90, 92, 121, 157 323)

Development of improved therapy for small cell lung cancer
(#66, 69, 78, 80, 85, 95, 103, 105, 108, 124, 177)

Development of improved therapy for ovarian cancer
(#99, 102, 125)

Development of improved methods in detecting and treating extragonadal germ cell tumors and other poorly differentiated tumors
(#22, 87, 93, 122, 123, 227, 228; 5-Book)

Use of cryopreserved cells for standardized immunological testing
(#37, 38, 40, 41, 45, 49, 52, 120)

Cancer Biotherapy: first to use the term to describe a fourth modality of cancer treatment
(#109, 114, 119, 128, 129, 134, 137, 139, 141, 146, 151 153, 154, 161, 169, 173, 174, 175, 176, 179, 181, 183,184, 188, 193, 197, 204, 206, 212, 225, 240, 249, 250,184, 188, 193, 197, 204, 206, 212, 225, 240, 249, 250,252, 260, 261, 262, 264, 265, 266, 267, 301, 302, 314,319, 322, 324, 325, 332, 333, 338, 339, 340, 343, 344, 346, 349, 356, 357, 358, 360, 361, 362, 363)10 Books)

Monoclonal antibodies and immunoconjugates in cancer treatment: Did groundbreaking experiments with toxin, drug and isotope conjugates
(#125, 126, 140, 143, 144, 145, 150, 152, 155, 156, 159, 160, 165, 180, 181, 182, 185, 187, 195, 196, 198, 199, 206, 218, 223, 224, 235, 238, 241, 244, 246, 247, 248, 251, 253, 254, 263, 278, 279, 285, 286, 287, 288, 291, 306, 307, 310, 311, 318, 325, 327, 342, 347)

Cytokine and Cellular Therapy: Conducted some of initial clinical experiments with IL-2 in LAK and T cell therapy
(#249, 255, 259, 281, 290, 294, 295, 298, 301, 302, 309, 311, 314, 322,324,332,333,349,356,357, 358, 361, 362)

Individualized Cancer Treatment: Conducted specific laboratory and clinical experiments demonstrating that each cancer in each patient has unique characteristics which may require unique, individualized treatment
(#267, 279, 285- 288, 291, 306, 307, 310, 318, 325, 327, 342, 347)

Privatization of Cancer Research: Brought attention to clinical research opportunities in the private sector. Published widely on problems of access to new technologies and the funding of clinical research
(#134, 205, 206, 207, 231, 236, 245, 256, 257, 268, 269,282, 284, 289, 293, 308, 312, 316, 317, 319, 321, 325, 326, 330, 331, 334, 335, 350, 352, 353, 354, 355, 358,359, 364, 11-Books)

Publications

Over 380 papers published in the medical/scientific literature

Thousands of abstracts, posters and presentations at various meetings on cancer research and treatment

Editor of Principles of Cancer Biotherapy, the first comprehensive textbook on the fourth modality of cancer treatment (Now in Third Edition).

Founding Editor of Cancer Biotherapy, (now Cancer Biotherapy & Radiopharmaceuticals), Molecular Biotherapy and the Journal of Biological Response Modifiers (now Journal of Immunotherapy).

Author or editor of thirteen books on cancer research and treatment

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Published Papers

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2 Oldham, R K Aseptic meningitis following the intrathecal injection of RISA Radiology, 97:317, 1970

3 Oldham, R K Eosinophilic Granuloma South. Med J , 64 978, 1971

4 Oldham, R K and Pomeroy, T C. Vincristine induced inappropriate ADH syndrome. South Med.J , 65 1010-1012, 1972

5 Oldham, R K and Pomeroy, T C Treatment of Ewing's sarcoma with Adriamycin Cancer Chemotherapy Reports, 56 635-639, 1972

6 Oldham, R.K and Pomeroy, T C Extramedullary plasmacytomas following successful radiotherapy of Hodgkin's disease Clinical and immunological aspects American J. Med., 54 761-767, 1973.

7 Oldham, R.K , Larson, S M., and Givelber, H.M , Chretien, P B , and Johnson, R E. A preliminary study of 51Cr-labeled platelets for evaluation of splenic sequestration in chronic lymphcytic leukemia J Nuclear Med , 37,219-222, 1973

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10 Lemevel, B P . Oldham, R K., Wells, S A , and Herberman, R B. An evaluation of 125Iododeoxyuridine as a cellular label for in vitro assays - Kinetics of incorporation and toxicity J Nat Cancer Inst , 51 1511-1558, 1973

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14 Mathe, G. and Oldham, R.K. Introducuon In Mathe, G. and Oldham, R.K. (Eds.): Complications of Cancer Chemotherapy. New York, Springer-Verlag, pp. 1-2, 1974

15 Beard, J., Weiner, R.S., Oldham, R.K., and Mathe, G.: Immune responsiveness in acute lymphocytic leukemia patients under chemotherapy and immunotherapy. A preliminary report. In Mathe, G. and Oldham, R.K. (Eds.): Recent Results in Cancer Research: Complications of Cancer Chemotherapy. New York, Springer-Verlag, pp 56-60, 1974

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PATENT
Docket No. 2043720003?0

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on August 25, 1997.

8-27-97 Alexandra M. Passons
Date Alexandra M. Passons

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF LYNN E. SPITLER, M.D.
PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Lynn E. Spitler, declare as follows:

1. I am a coinventor of the subject matter in the above-referenced application. I have been a practicing immunologist for many years. A copy of my *curriculum vitae* is attached hereto as Exhibit A.

2. I have been managing clinical trials of OncoVax®™ prostate cancer vaccine which is recombinant human prostate specific antigen (rhPSA) formulated in liposomes with lipid A as adjuvant. Lipid A is a preferred adjuvant because of its ability to stimulate cell-mediated immunity in general and production of cytotoxic T cells (CTL) in particular, as opposed to alum which enhances the antibody response.

3. We conducted four clinical Phase I/II trials with 4 or 5 patients in each trial. In the first two trials, eligible patients had surgically incurable and hormone-resistant prostate cancer as

well as intact immune responses as measured by ability to respond to recall antigens. Patients were given 1 ml of the vaccine containing 100 µg rhPSA 25 and 200 µg lipid A. In the first trial, the vaccine was administered intramuscularly in two divided doses on two extremities with intact regional lymph nodes every 30 days times 3. In this trial, one patient showed skin test sensitivity to PSA with a diameter of erythema of 18 mm, and a diameter of induration of 10 mm (when diameters of more than 5 mm are considered positive responses).

4. In the second trial, the same dosage and regimen was used except that an intravenous route was employed. Again, one of five patients showed reactivity to a skin test with PSA with a diameter of erythema of 10 mm.

5. In a third trial, the adjuvant GM-CSF was added to the protocol and the vaccine was administered with the same protocol intramuscularly. In this test, all 4 evaluable vs patients showed skin test reactivity to PSA with a mean diameter of erythema of 37 mm. One patient had an erythema diameter of 70x50 mm.

6. In the fourth trial, patients were treated with cyclophosphamide to diminish suppressor cell activity and the vaccine was administered intracutaneously with the addition of *Bacillus Calmette-Guerin* (BCG) at the site of vaccine administration. In this trial, 4 of the 5 patients showed a skin response to PSA with a mean diameter of erythema of 17 mm and a mean induration diameter of 9 mm.

7. The data are shown in detail on attached Exhibit B. These results show that administration of OncoVax-P™ is successful in eliciting a cellular response in these patients, especially in the presence of a suitable adjuvant.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at Tiburon, California on 26 August 1997, by

Lynn E Spitler

Lynn E. Spitler, M.D.

dc-85047

** TOTAL PAGE.08 **

CURRICULUM VITAE

LYNN E. SPITLER, M.D.

1895 Mountain View Drive
Tiburon, California 94920

(415) 435-9861
FAX (415) 435-6851

Social Security #366-38-1697

Education:

University of Michigan, Ann Arbor, Michigan	1956-1959
University of Michigan Medical School Ann Arbor, Michigan M.D. (cum laude)	1959-1963

Training:

Intern	Highland Alameda County Hospital Oakland, California	1963-1964
Resident	University of California School of Medicine San Francisco, California	1964-1966
Research Fellow	H.S. Lawrence, M.D. Department of Medicine - Immunology New York University New York, New York	1966-1967
Immunology Trainee	H. Hugh Fudenberg, M.D. University of California School of Medicine San Francisc . California	1967-1969

Teaching Appointments and Employment:

Instructor of Medicine in Residence University of California School of Medicine San Francisco, California 94143	1970-1971
Assistant Professor of Medicine in Residence University of California School of Medicine San Francisco, California 94143	1971-present
Research Associate Cancer Research Institute University of California School of Medicine San Francisco, CA 94143	1971-1978
Director, Melanoma Center Northern California Health Center San Francisco, California 94118	1978-1990
Director of Research Children's Hospital of San Francisco San Francisco, California 94118	1978-1981
Member, Graduate Group in Comparative Pathology Department of Comparative Pathology University of California, Davis Davis, California	1976-1981
Senior Vice President XOMA Corporation 2910 Seventh Street Berkeley, California 94710	1981-1988
Associate Scientific Director Biotherapeutics, Inc. 357 Riverside Drive Franklin, Tennessee, 37065-1676	1988-1989

Director — 1990-present
Northern California Melanoma Centers
1895 Mountain View Drive
Tiburon, California 94920

President — 1991-present
Jenner Technologies
1895 Moountain View Drive
Tiburon, California 94920

Awards and Honors:

Recipient: Dernham Senior Fellowship California Div. of the American Cancer Society	1969-1971
Recipient: Research Career Development Award National Institutes of Health	1971-1976
Alpha Omega Alpha (Junior year)	1961
Outstanding Young Women of America	1968
Who's Who of American Women	1972
Who's Who in the West	1973

Board Certification:

American Board of Internal Medicine	1972
American Board of Allergy and Immunology	1974

Licensure:

Michigan	25985
New York	96454
Calif rnia	C-26446

Memberships in Professional Societies:

American Association of Immunologists
American Association for the Advancement of Science
Alpha Epsilon Iota
Western Society for Clinical Research
American Federation of Clinical Research
Society of Biological Therapy
American Association for Cancer Research

Patents:

Patent #4,489,810 for "Composition and Method for Transplantation Therapy"

Patent #4,590,071 for "Human Melanoma Specific Immunotoxins"

PUBLIC SERVICE

National Review Committees:

Allergy and Immunology Research Committee, NIAID, NIH	1976-1980
Merit Review Board in Immunology, VA, Washington, D.C. (Chairman 1979-1980)	1976-1980

Editorial Boards:

The Journal of Immunology	1975-1978
The International Journal of Immunopharmacology	1979-1984
Immunologia Clinica e Sperimentale	1982-1986
Antibody Immunoconjugates and Radiopharmaceuticals (Associate Editor)	1987-present
Molecular Biotherapy	1987-present
Cancer Biotherapy	1991-present

Manuscript Reviews:

American Review of Respiratory Disease
Annals of Allergy
Annals of Internal Medicine
Archives of Dermatology
Archives of Internal Medicine
California Medicine
Cancer
Cancer Immunology and Immunopathology
Cancer Research
Cellular Immunology
Chest
Infection and Immunity
Infectious Disease and Immunology
International Journal of Immunopharmacology
Journal of Clinical Investigation
Journal of Immunology
Journal of Infectious Diseases
Journal of the American Academy of Dermatology
Molecular Biotherapy
Nature
New England Journal of Medicine
Science
The Western Journal of Medicine

Special Consultant:

National Institutes of Health Grant Reviews
National Institutes of Health Site Visits
United States Tuberculosis Panel Task Group
Atomic Energy Commission Site Visits
Public Education Panel, National Multiple Sclerosis Society
Review of Grant Applications for the National Science Foundation
Enterprise for High School Students, Medical Apprenticeship Program, San Francisco, California
Board of Directors, San Francisco Unit, American Cancer Society
Research and Human Experimentation Committee, Children's Hospital f San Francisco
U.S. Energy Research Development Administration Site Visits

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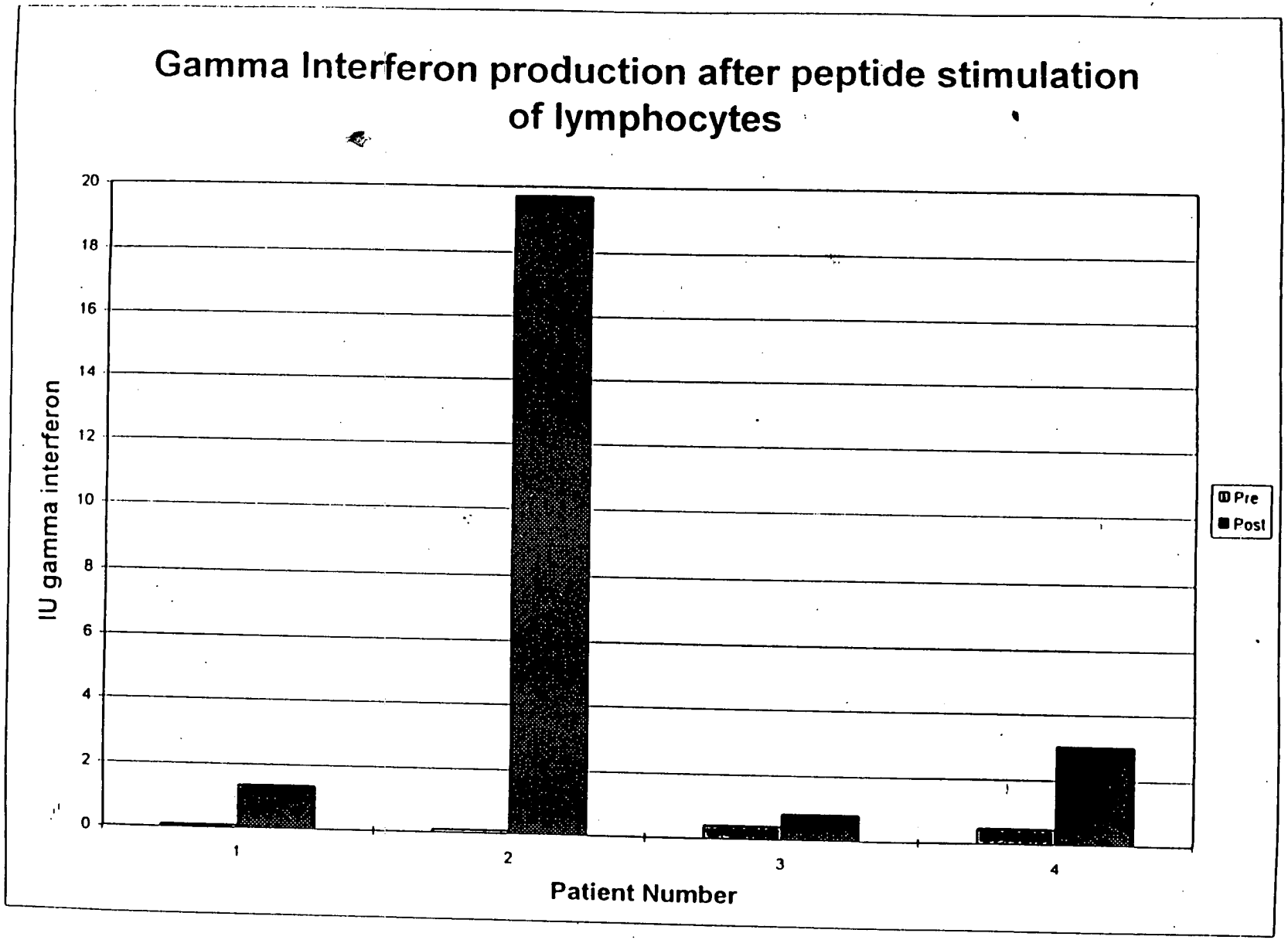
Gamma Interferon production after peptide stimulation of lymphocytes
IU gamma interferon
20
18
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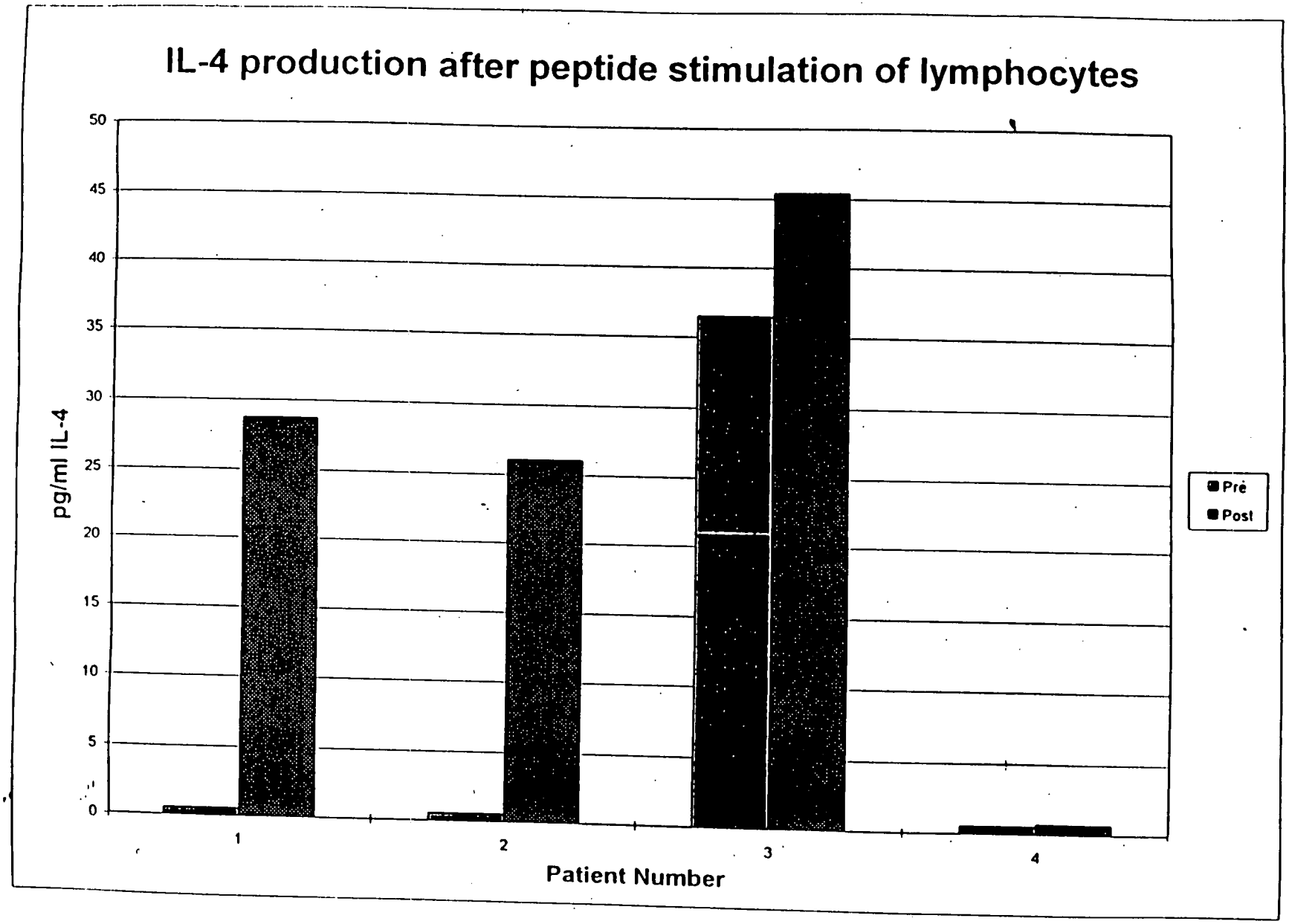
Patient Number
Pre
Post

Exhibit B


IL-4 production after peptide stimulation of lymphocytes
pg/ml IL-4
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45
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1
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3
4
Patient Number
Pre
Post

Exhibit B

Prostate Cancer Vaccine Trials
Day 90 Skin Test Results

Clinical Trial #1 – Immunization of Patients with Prostate Cancer with OncoVax-P™ (Prostate Cancer Vaccine)

	PSA		Buffer		CEA	
	Erythema	Induration	Erythema	Induration	Erythema	Induration
001 AW	2x2	0	0	0		
002 JH	ND	ND	ND	ND		
003 MD	18x18	10x10				
004 MED	0	0	0	0		
005 HN	0	0	0	0		
006 JLB	0	0	0	0		

Clinical Trial #2 – Intravenous Immunization of Patients with Prostate Cancer with OncoVax-P™ (Prostate Cancer Vaccine)

	PSA		Buffer		CEA	
	Erythema	Induration	Erythema	Induration	Erythema	Induration
101 RT	0	0	0	0		
102 ER	0	0	0	0		
103 MC	0	0	0	0		
104 TM	0	0	0	0		
105 AF	ND	ND	ND	ND		
106 WC	10X10	0	0	0		

Clinical Trial #3 –Immunization of Patients with Prostate Cancer with OncoVax-P™ (Prostate Cancer Vaccine) with GM-CSF

	PSA		Buffer		CEA	
	Erythema	Induration	Erythema	Induration	Erythema	Induration
201 AM	30x20	0			0	0
202 ASP	35x30	30x30			7x7	7x7
203 CN	70x50	0			0	0
204 JA	ND	ND			ND	ND
205 GS	37x22	0			0	0

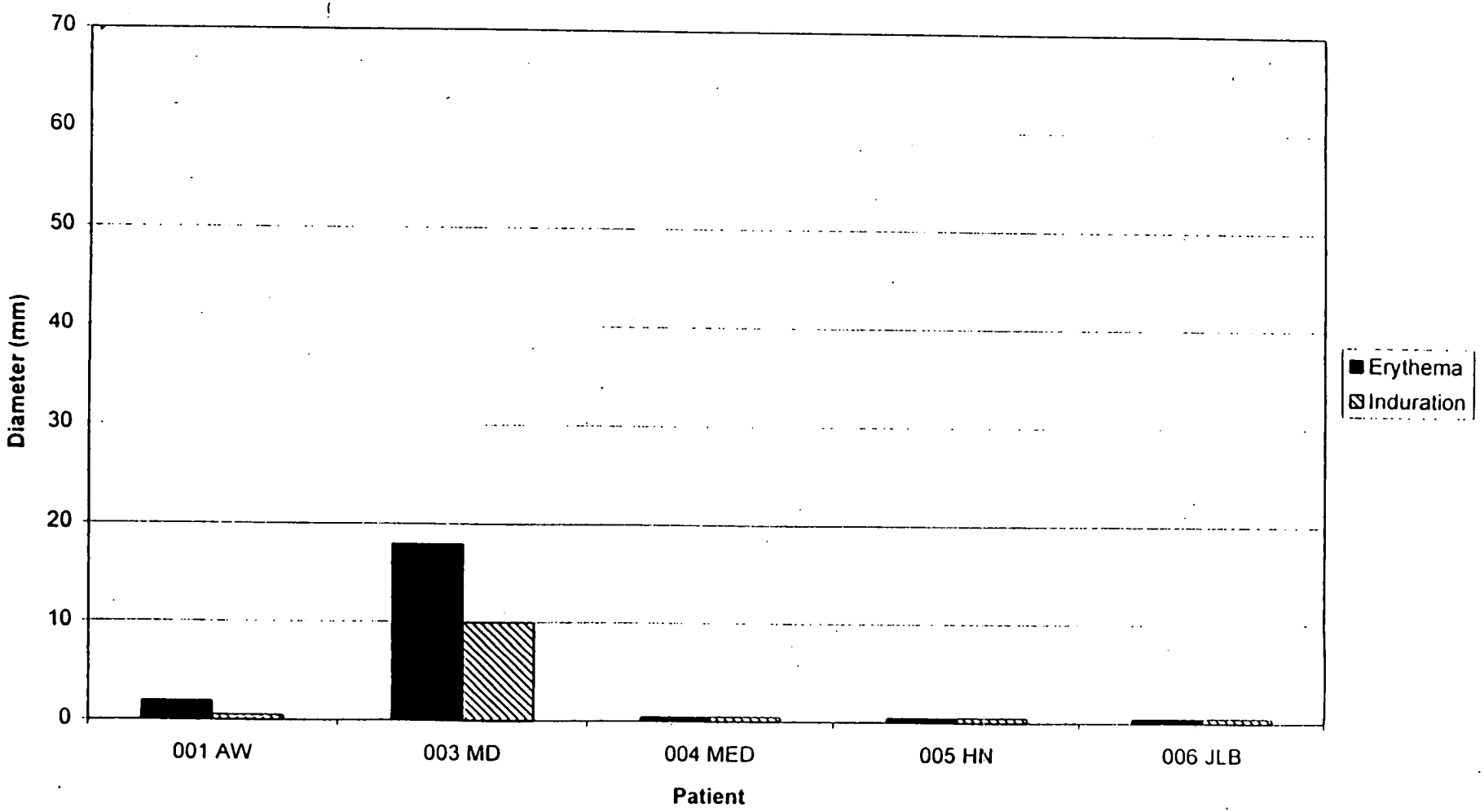
Clinical Trial #4 – Immunization of Patients with Prostate Cancer with OncoVax-P™ (Prostate Cancer Vaccine with BCG

	PSA		Buffer		CEA	
	Erythema	Induration	Erythema	Induration	Erythema	Induration
301 GS	32x28	32x28			0	0
302 AR	15x15	15x15			0	0
303 JE	15x12	0			0	0
304 ML	0	0			0	0
305 RD	30x30	0			0	0

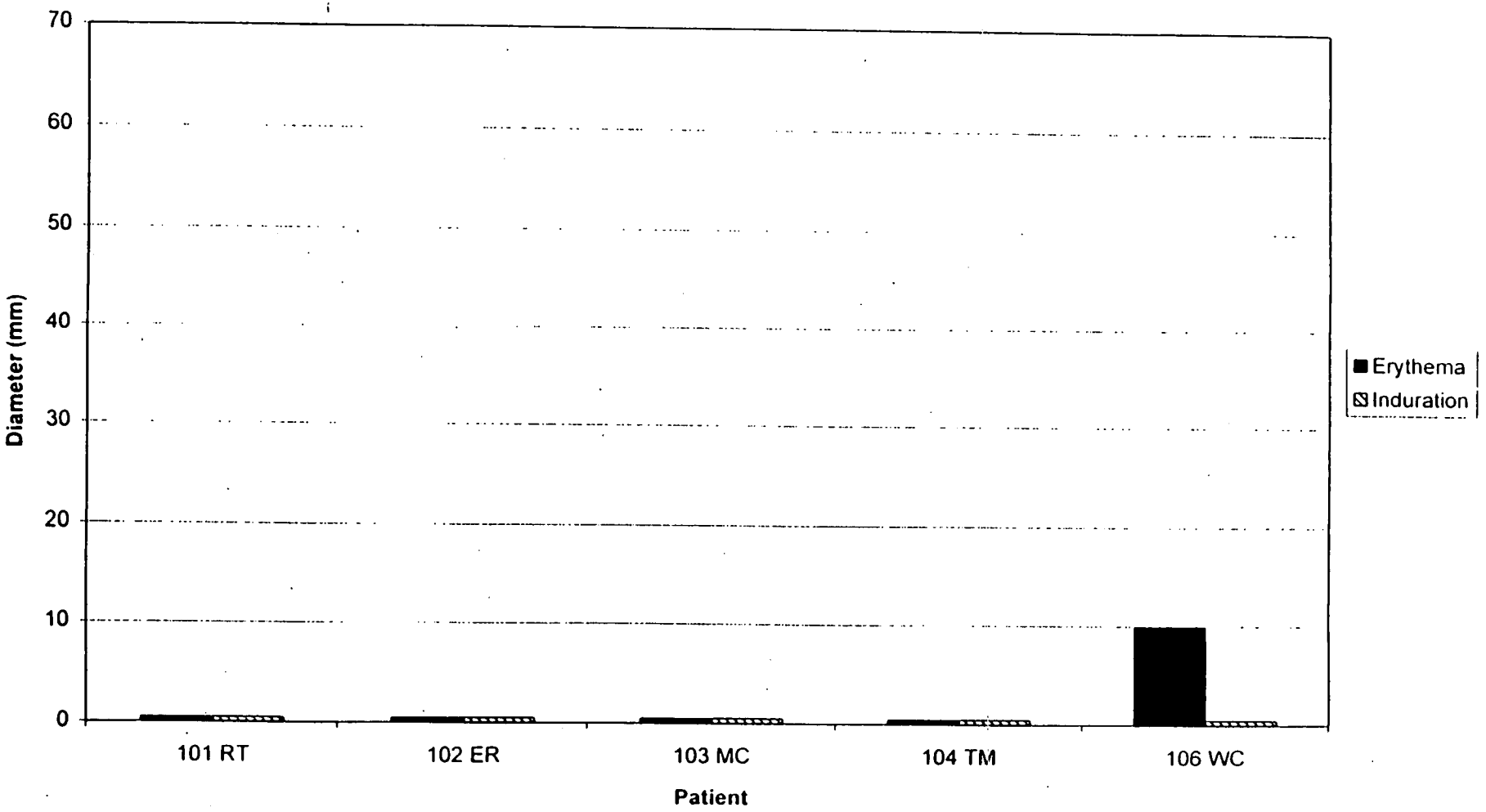
ND = Not Done

Exhibit B
Spitler

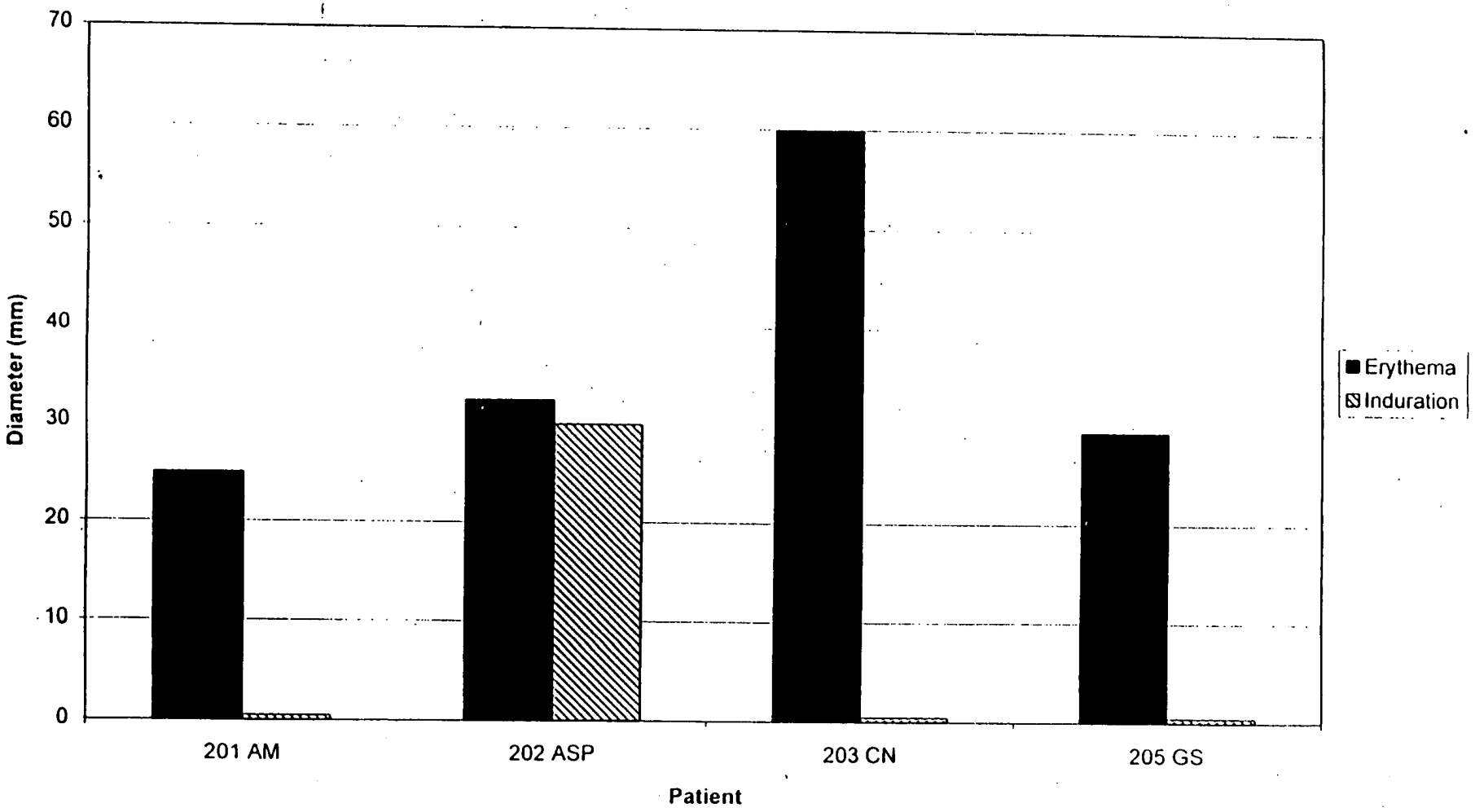
Skin Test Reactivity to PSA
Clinical Trial #1
Immunization of Patients with Prostate Cancer with OncoVax-P™



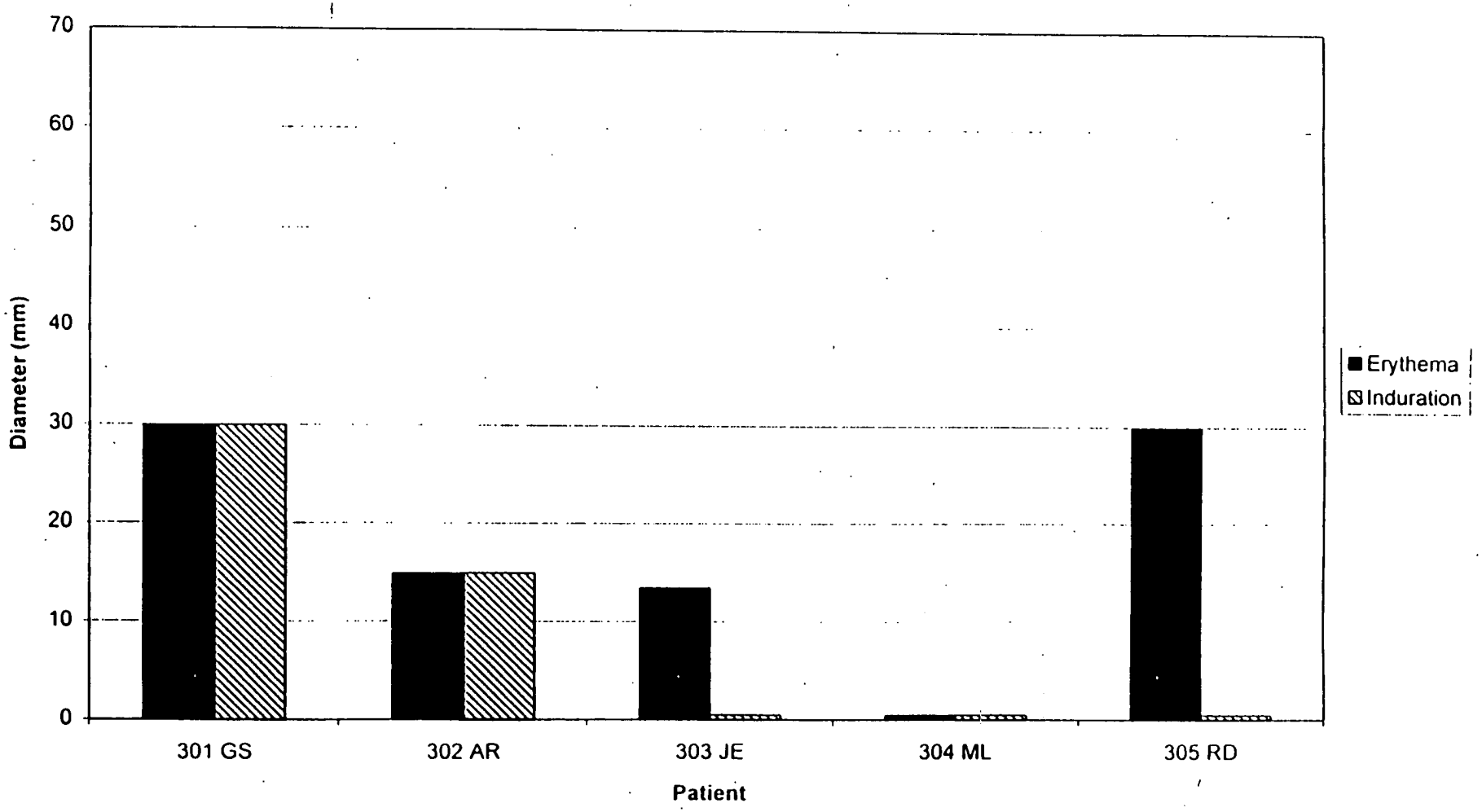
Skin Test Reactivity to PSA
Clinical Trial #2
Intravenous Immunization of Patients with Prostate Cancer with OncoVax-P™



Skin Test Reactivity to PSA
Clinical Trial #3
Immunization of Patients with Prostate Cancer with OncoVax-P™ with GM-CSF



Skin Test Reactivity to PSA
Clinical Trial #4
Intravenous Immunization of Patients with Prostate Cancer with OncoVax-P™ with BCG



PATENT
Docket No. 204372000320

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

8-27-97 Alexandra H Parsons
Date Alexandra H. Parsons

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF GARY R. MATYAS, PH.D.
PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Gary R. Matyas, declare as follows:

1. I am engaged in medical research at the Walter Reed Army Institute of Research (WAIR) and have been active in studying cellular immune responses for 2 years. A copy of my *curriculum vitae* is attached hereto as Exhibit A.

2. Under my supervision, studies were performed using Balb/C mice. In order to generate cytotoxic T lymphocytes (CTL) in response to recombinant human prostate specific antigen (rhPSA) we immunized two groups of six mice per group with OncoVaxP™ alone or with OncoVaxP™ mixed with aluminum hydroxide (alum, Alhydrogel, 200 μg Al^{+++}).

OncoVaxP™ is a liposomal formulation of rhPSA with lipid A, and the PSA dose in this protocol was 5 μg rhPSA and 20 μg lipid A. The mice were immunized at week 0 and week 4 at these dosages and euthanized at 8 weeks. The spleens were removed and spleen cells harvested, pooled and incubated for 5 days either with medium alone or in medium containing 2 μg/ml rhPSA.

3. Target cells were P815 mouse mastocytoma cells infected either with wild-type vaccinia virus or with vaccinia virus transfected with the PSA gene and the *E. coli* lacZ gene (PSAVac, Therion Biologics Corp.) at a multiplicity of infection of 10. Alternatively, the P815 cells were incubated with 10 μg/ml of the peptide CYASGWGSI which represents a potential CTL epitope at positions 153-161 of PSA.

4. After incubation for 16 hours with vaccinia viruses or peptide, the target cells were labeled with Cr^{51} (0.1 μCi/10^6 cells) for 1 hour).

5. The effector cells harvested from the moue spleens were plated in 96-well u-bottom plates and the Cr^{51} labeled target 815 cells were added at various effector:target ratios. The plates were centrifuged at 50xg for 5 minutes and then incubated at 37°C for 5 hours. The radioactivity present in the supernatant (indicative of cytotoxic activity of the effector cells) was harvested using Skatron wicks and quantified using a gamma counter.

6. The attached Exhibit B shows the results. As shown, spleen cells from mice injected with either OncoVaxP™ or OncoVaxP™ with alum were able to lyse target cells that had been transfected with the vaccinia virus (Panels A and B). OncoVaxP™ injected mice in the absence of alum (panel E) were effective in lysing the targets labeled with peptide, although when alum was included in the formulation, the resulting spleen cells were less able to do so (panel F). P815 target cells unlabeled with either peptide or vaccinia-produced PSA were not lysed (panels C, D, G, and H).

7. These results demonstrate that immunization of mice with OncoVaxP™ induces lymphocytes which kill tumor cells presenting PSA antigens, indicating an antitumor response.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at Washington, D.C. on 26 August 1997, by

Gary R. Matyas

August . , 1997

CURRICULUM VITAE

Gary R. Matyas

Office:
Department of Membrane Biochemistry
Walter Reed Army Institute of Research
Washington DC 20307-5100
(202) 782-0875

Home:
18415 Snowberry Way
Olney, MD 20832
(301) 570-0610

CAREER AIMS: Research/management of membrane biochemistry laboratory with emphasis on liposome based vaccines, lipid mediators, and lipid changes during cell growth.

PERSONAL: Born April 30, 1956 in Berwick, PA; married, one child

EDUCATION:

Graduate
Purdue University, West Lafayette, IN 47907
Ph.D. degree; May 1985; Department of Biological Sciences
Major Professor: D. James Morré

Undergraduate
Pennsylvania State University, University Park, PA 16802
B.S. degree; May 1978; Major - Biophysics

EXPERIENCE:

Research
Research Chemist
Department of Membrane Biochemistry
Division of Biochemistry
Walter Reed Army Institute of Research
Washington DC 20307
September 1988 to present
Supervisor: Dr. Carl R. Alving

Projects:

I. Development of active site monoclonal antibodies to cobra venom phospholipase A_2: Inhibitors of enzyme activity

WRAIR Projects (cont.):

II Mechanism of concanavalin A induced killing of mice and cultured cells

II Incorporation of bioactive lipids into liposomes: Effect on immune response to liposome encapsulated antigens

IV Development of monoclonal antibodies to sphingosine and sphingolipids

V Development of a liposomal vaccine against ricin intoxication

VI In collaboration with Jenner Technologies, development, manufacture and testing of liposomal based cancer vaccine in human clinical trials

VII Development of a liposomal vaccine protects against Ebola virus infection through the induction of cytotoxic lymphocytes.

Additional Duties:

I. Division of Biochemistry Safety Officer, Division representative to WRAIR safety council; Responsibilities include: Monthly safety inspections of division laboratories; Conducting training on safety concerns; Maintaining division training records.

II. Terminal Security Officer for the Department of Membrane Biochemistry; Duties include: Maintaining security on computer equipment in the department; Procuring of IBM compatible equipment and software.

Staff Fellow
Membrane Biochemistry Section
Laboratory of Molecular and Cellular Neurobiology/Developmental and Metabolic Neurology Branch
National Institute of Neurological, Communicative Disorders and Stroke
National Institutes of Health
Bethesda, MD 20892
April 1985 to September 1988
Supervisor: Dr. Peter Fishman

Projects:

I. Glycolipid alterations induced by transfection of NIH/3T3 cells with oncogenes.

II. Alterations of glycolipids during cell growth.

NIH projects (cont.)

II Production of glycolipid crosslinking reagents for the study of the involvement of glycolipids in cell adhesion and cell growth.

IV. The role of *ras* oncogenes in phospholipase C mediated phosphoinositide hydrolysis.

Graduate Assistant
Department of Biological Sciences
Purdue University
West Lafayette, IN 47907
August 1978 to April 1985
Supervisor: Dr. D. James Morré

Projects:

I. Interaction of fibronectin with gangliosides.

II Subcellular distribution and biosynthesis of gangliosides in rat liver.

III. Loss of fibronectin and complex gangliosides in metastatic rat tumors.

IV. Elevated levels of serum gangliosides as a means of early detection of cancer.

V. Cytochemical localization of glycosyltransferases.

Research Assistant
Department of Biochemistry and Biophysics
Pennsylvania State University
University Park, PA 16802
September 1977 to May 1988
Supervisor: Dr. Wallace Snipes

Project:

I. Inactivation of lipid-containing viruses through physical perturbation of membranes.

Teaching

Instructor
Introductory Microbiology Laboratory
Department of Biological Sciences
Purdue University
West Lafayette, IN 47907
January 1981 to May 1981
Supervisor: Dr. David Filmer

Teaching Assistant
Introductory Microbiology Laboratory
Department of Biological Sciences
Purdue University
West Lafayette, IN 47907
August 1979 to May 1980, August 1978 to May 1979
Supervisors: Dr. Allen Konopka and Dr. David Filmer

AWARDS:

Special Purdue Fellowship
Purdue University
West Lafayette, IN 47907
August 1980 to December 1980; $2,000

Marion County Cancer Society (Little Red Door) Fellowship
1801 North Meridian Street, Indianapolis, IN 46202
"A New Serodiagnostic Parameter for Early Detection of Cancer That May Distinguish Localized and Disseminated Disease"
January 1982 to December 1982; $12,498

Milheim Foundation for Cancer Research
Colorado National Bank
Seventeenth Street at Champa
Denver, CO 80202
"The Biochemical Basis for Metastasis"
July 1982 to June 1983; $11,421

David Ross Fellowship
Purdue University
West Lafayette, IN 47907
"Protein Kinase Modulations as Early Events of Tumorigenic Progression"
August 1983 to July 1985; $13,200

MEMBERSHIPS:

American Association for the Advancement of Science
1978 to present

American Society for Biochemistry and Molecular Biology
1987 to present

American Society of Microbiology
1996 to present

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Gary R. Matyas

PUBLICATIONS:

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2. Matyas, G. R. and Morre, D. J.: Coupling of Uridine-5'-Diphosphate (UDP) Formation and Nicotinamide Adenine Dinucleotide (NADH) Reduction for Cytochemical Localization of Glycosyltransferases. Journal of Histochemistry and Cytochemistry (1983) 31, 1175-1182.

3. Matyas, G. R. and Morre, D. J.: Isolation of Purified Membranes and Membranous Cell Components for Receptor Studies. Investigations of Membrane-Located Receptors (Methodological Surveys (B): Biochemistry Vol. 12, Eds. Reid, E., Cook, G. M. W. and Morré, D. J., Plenum Publishing Corp., New York, NY (1984) pp. 111-118.

4. Morre, D. J., Creek, K. E., Matyas, G. R., Minnifield, N., Sun, I., Baudoin, P., Morré, D. M. and Crane, F. L.: Free-flow Electrophoresis for Subfractionation of Rat Liver Golgi Apparatus. Biotechniques (1984) 2, 224-233.

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13. Matyas, G. R., and Fishman, P. H.: Lipid Signalling Pathways in Normal and *Ras*-Transfected NIH/3T3 Cells. Cellular Signalling (1989) 1, 395-404.

14. Buckley, N. E., Matyas, G. R. and Spiegel, S.: The Bimodual Growth Response of Swiss 3T3 Cells to the B Subunit of Cholera Toxin Is Independent of the Density of Its Receptor, Ganglioside, GM1. Experimental Cell Research (1990) 189, 13-21.

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19. Carl R. Alving, Glenn M. Swartz, Nabila M. Wassef, Jorge L. Ribas, Edward E. Herderick, Renu Virmani, Frank D. Kolodgie, Gary R. Matyas, and Julie F. Cornhill. Immunization with Cholesterol-Rich Liposomes Induces Anti-Cholesterol Antibodies and Reduces Diet-Induced Hypercholesterolemia and Plaque Formation. Journal Laboratory and Clinical Medicine (1996) 127:40-49.

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MANUSCRIPTS SUBMITTED:

1. Gregory M. Glenn, Mangala Rao, Gary R. Matyas, Patricia Walker and Carl R. Alving. Transcutaneous Immunization Using Bacterial ADP-Ribosylating Exotoxin. Science (submitted).

2. Mangala Rao, Gary R. Matyas, Franziska Grieder, Kevin Anderson, Peter B. Jahrling, and Carl R. Alving. Cytotoxic T Lymphocytes to Ebola Zaire Virus Are Induced in Mice by Immunization with Liposomes Containing Lipid A. European Journal of Immunology (submitted).

ABSTRACTS:

1. Matyas, G. R. and Werderitsh, D. A.: Comparison and Ganglioside Composition of Metastatic and Non-metastatic Transplantable Tumors from the Jaw Region of the Rat. Federation Proceedings (1981) 40, 1544.

2. Morré, D. J., Hsu, H., Matyas, G. R., Kloppel, T., Classen, P. and Jacobson, W.: Lipid-associated Sialic Acid (LASA) Elevated in Sera of Carcinoma Patients. Proceedings of the American Association of Cancer Research (1981) 22, 22.

3. Morgan, D. E., Morré, D. M., and Matyas, G. R.: Effects of Vitamin A on Various Hematological Parameters of Rats Bearing Tumors. Proceedings of the Indiana Academy of Science (1981) 91, 147.

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26. Matyas, G. R. and Alving, C. R.: Effect of Route of Administration on Generation of Different Isotype Antibodies to Liposome-Encapsulated Ricin Subunits. The FASEB Journal (1994) 8, A1464.

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Gary R. Matyas

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INDUCTION OF CYTOTOXIC T-LYMPHOCYTES TO TUMOR CELLS PRESENTING PROSTATE SPECIFIC ANTIGENS

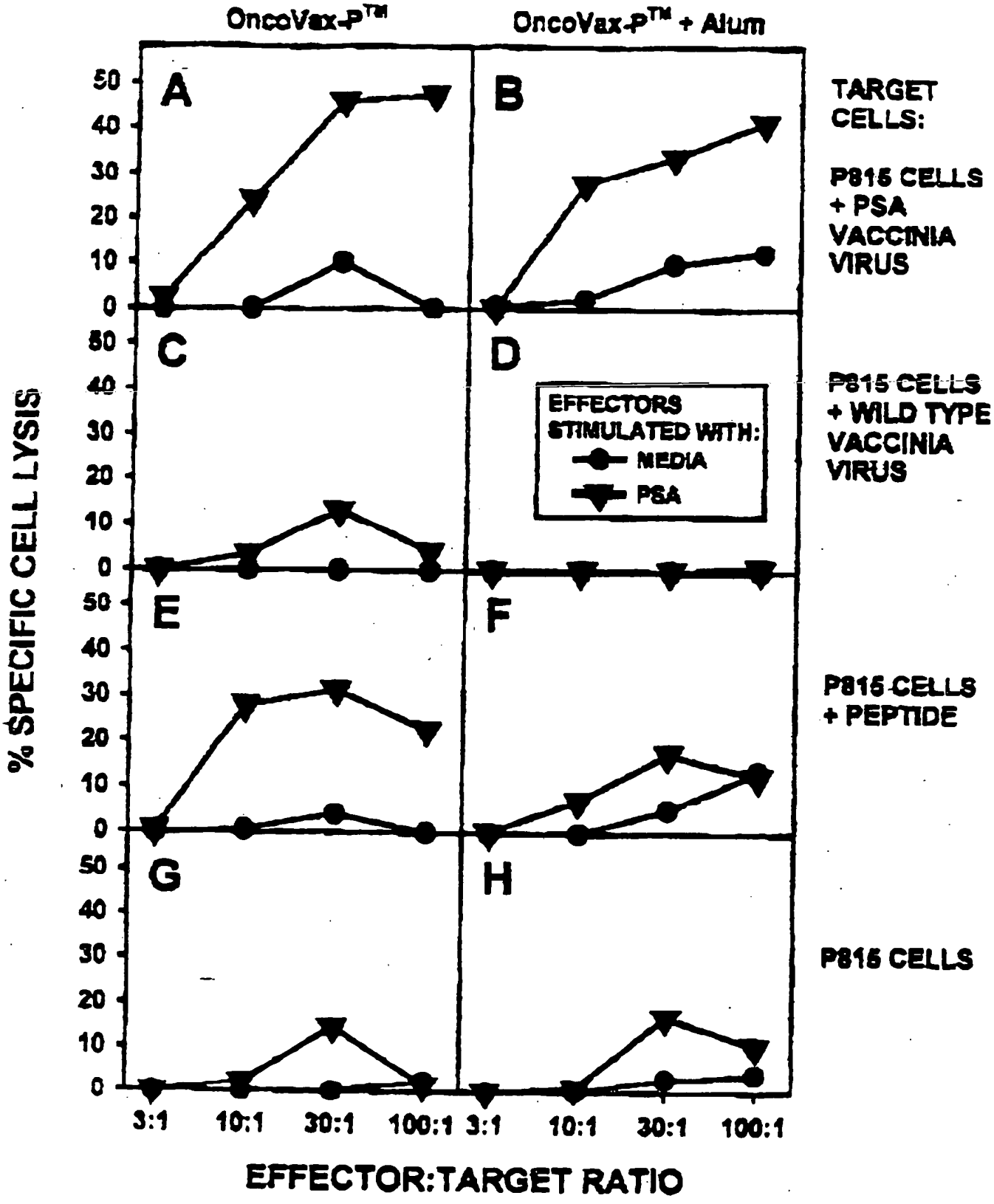


Exhibit B

Matyas

Induction of Antitumor Response in Mice by Prostate Cancer Vaccine

Gary Matyas. PhD, Mangala Rao, PhD, Jean Muderhwa, PhD, and Carl Alving,MD, Walter Reed Army Institute of Research, Washington, DC

We report that immunization of mice with OncoVax-P™ prostate cancer vaccine induces cytotoxic T-lymphocytes which have the capability of killing tumor cells presenting PSA antigens.

Two groups of Balb/c mice (6 mice/group) were immunized with OncoVax-P™. One group was immunized with OncoVax-P™ alone and the other group received OncoVax-P™ mixed with aluminum hydroxide (alum, Alhydrogel) (200 µg Al^{+++}). OncoVax-P™ is a liposomal formulation of prostate specific antigen (PSA) with lipid A, as an adjuvant. The PSA dose was 5 µg PSA and 20 µg of lipid A. The mice were immunized at week 0 and 4 weeks after the boost. Three animals per group were euthanized 4 weeks after the boost and the spleens were removed for CTL assay. The cells from each group of spleens were pooled and incubated 5 days with media alone or 2 µg/ml human PSA. Target P815 cells (mouse mastocytoma) were infected with either wild type vaccinia virus (WTvac) or with vaccinia virus transfected with PSA gene and the *E. coli* lac Z gene (PSAvac, Therion Biologics Corporation) at an multiplicity of infection of 10. P815 cells were incubated with 10 µg/ml of peptide, CYASGWGSI, which represents a potential murine CTL epitope for PSA. It was identified using the University of Wisconsin Genetics Computer Group sequence analysis finding pasterns computer program. This program was used to compare the amino acid sequence of PSA with the known murine CTL anchor sequences. CYASGWGSI is located at amino acid positions 128-137 of PSA. The P815 targets were incubated for 16 hours with vaccinia viruses or peptide. The targets cells were then labeled with Cr^{51} (0.1 µCi/10^6 cells) for one hour. Effector cells were harvested and plated in 96 well U bottom plates. Cr^{51} labeled targets were added at various effector:target ratios. Following centrifugation at approximately 50 X g for 5 min, the plates were incubated at 37°C for 5 hr. The radioactivity present in the supernatant was harvested using Skatron wicks and the radioactivity was quantified using a gamma counter.

As shown in the attached figure. murine CTL which specifically lysed PSAvac infected targets (A & B) and targets incubated with peptide (E) were obtained. Approximately, 47% cell lysis was obtained using PSAvac targets. WTvac targets were not lysed (C & D), indicating the CTLs were specific for PSA epitopes. Addition of alum to the vaccine had no effect on the generation of CTL which lysed PSAvac targets (A & B) but it inhibited the induction of CTLs to tumor cells pulsed with peptide.

These results indicate that immunization of mice with the prostate cancer vaccine induces lymphocytes capable of specifically killing tumor cells presenting PSA antigens indicating an antitumor response.

PATENT
Docket No. 204372000320

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on April __, 1998.

Date

Alexandra H. Parsons

hand delivered 4/29/98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. Spitler et al.

Serial No.: 08/288,057

Filing Date: 10 August 1994

For: PROSTATIC CANCER VACCINE

Examiner: P. Gambel

Group Art Unit: 1816

DECLARATION OF LYNN E. SPITLER, M.D.
PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Lynn E. Spitler, declare as follows:

1. I am a coinventor of the subject matter in the above-referenced application and have been a practicing immunologist for many years. My *curriculum vitae* is of record in this application.

2. I have been and am directing the clinical trials of OncoVaxP™, an immunological preparation for effecting an antitumor effect with regard to prostate cancer. OncoVaxP™ contains recombinant human prostate-specific antigen (rhPSA) formulated into compositions which are designed to behave as adjuvants. In a previous Declaration, executed 26 August 1997, I described the results of four clinical trials which showed promising results for the rhPSA as antigen. Exhibit B to my previously submitted Declaration summarized these results.

3. We have conducted an additional, fifth clinical trial in which the rhPSA was prepared in a liposomal formulation which was then emulsified with mineral oil. A group of 5 patients was treated in this study. They had undergone previous treatment for prostate-derived tumors. The attached Exhibit A contains a summary of the particulars relating to these patients. In 3 of the 5 patients the tumor had metastasized to the bone. The OncoVaxP™ vaccine was administered over the period of August 1997 to November 1997 or September 1997 to December 1997 as shown.

4. As in the previous four trials, several measures of immune response were obtained: skin test reactivity to PSA, production of IgG antibodies, and, most important, proliferation of lymphocytes in response to PSA. As shown in Chart 3 as part of Exhibit A, the 5 patients uniformly gave a dramatic response to OncoVaxP™ in the form of lymphocyte proliferation in response to PSA -- a result associated with the cellular immune system. In all cases, lymphocyte proliferation in response to PSA was dramatically improved after the vaccination protocol.

5. These results are highly indicative of an antitumor effect. The relationship between cell-mediated immunity and control of tumor growth is well documented. Cell-mediated immunity can be measured either by delayed type hypersensitivity (DTH) skin testing or by lymphocyte proliferation responses to the vaccine antigen. These are simply alternative assays for a cellular response. I have attached several examples of this documentation as Exhibits B, C, D and E.

6. In Exhibit B, a paper by Barth, A. *et al. Cancer Research* (1994) 54:3342-3345, discloses that melanoma patients were administered a polyvalent melanoma cell vaccine. The authors noted that overall survival was significantly prolonged in patients with positive DTH reaction and/or an increased cytotoxic T cell activity. For example, Figure 2 in Exhibit B shows a comparison of survival times of patients with DTH $\geq$ 6 mm compared to patients DTH < 6 mm. As shown, the survival time of the responders was dramatically increased ($p=0.0054$). The median survival was 52 months in the DTH-positive group as compared to 22 months in the DTH-negative group.

7. As shown in Exhibit C, an abstract of an article by Bystryn, J.C. *et al. Cancer* (1992) 69:1157-1164, the purpose of this study was to determine whether there was a relationship between induction of DTH in response to vaccination with a melanoma vaccine and

disease recurrence. Melanoma patients were administered a partially purified polyvalent melanoma antigen vaccine. The median disease-free survival of patients was correlated with their response to DTH. A statistically significant relationship was found. The median disease-free survival of patients with a strong DTH response was 4.7 years longer than that of those who did not respond.

8. In Exhibit D, an abstract of a paper by Berd, D. *et al. J Clin Oncol* (1997) 15:2359-2370, melanoma patients were administered autologous whole cells modified with the hapten dinitrophenyl (DNP). The authors noted that the development of a positive DTH response to the autologous melanoma cells which were unmodified with DNP was correlated with a significantly longer five-year survival.

9. Finally, Exhibit E is an abstract of an article by McCune, C.S. *et al. Cancer Immunol Immunother* (1990) 32:62-66. Patients with metastatic renal cell carcinoma were vaccinated with autologous tumor cells and skin-tested for an immunological response. The authors concludes that the survival times of the skin-test-positive group were significantly superior to those of the skin-test-negative group.

10. The articles cited are only four of many which show that a response by the cellular immune system to tumor-associated antigens is correlated with antitumor effect and clinical benefit as demonstrated by increased disease-free survival and/or survival time. Generally, it is recognized that this correlation is strong. Much of the difficulty in obtaining an immune response lies in the difficulty of assuring a uniform, potent composition to administer, a problem not encountered with rhPSA.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at Wash DC, ~~California~~ on April 29 1998, by

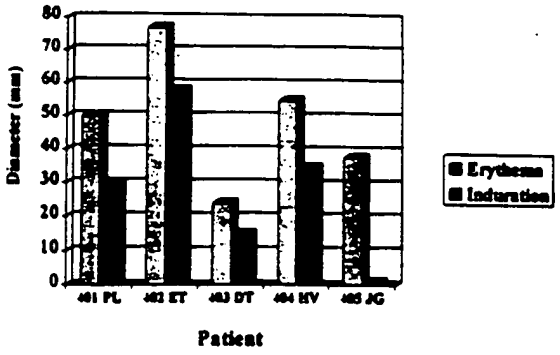
Lynn E. Spitler, M.D.

Clinical Trial #5
Administration of OncoVax-P™ Emulsion
February 6, 1998

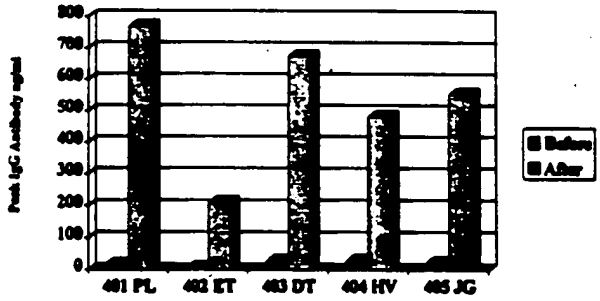
Patient	Age	Prior Treatment	Sites	Vaccine	PSA Pre	PSA D 90	Clin Resp	PSA Last	Post	Last FU	Death
1. PL (#401)	67	Biopsy Orchiectomy Hormonal	Prostate Bone	8/97 – 11/97 1/98	0.98	0.31	S	<0.05 (1/98)	Boost	1/98	
2. ET (#402)	70	Biopsy Hormonal	Prostate Bone	8/97 – 11/97 1/98	46.3	525.80	Improved Bone Scan*	1622.40 (1/98)	Boost	1/98	
3. DT (#403)	74	Biopsy Radiation Hormonal	Prostate	8/97 – 11/97 1/98	9.91	13.55	S	6.51 (1/98)	Boost	1/98	
4. HV (#404)	70	Biopsy Radiation Hormonal	Prostate Bone	9/97 – 12/97	18.13	51.46	S		Boost	12/97	
5. JG (#405)	75	Biopsy Radiation	Prostate	9/97 – 12/97	35.40	39.91	S			12/97	

*Alkaline phosphatase 1200→300

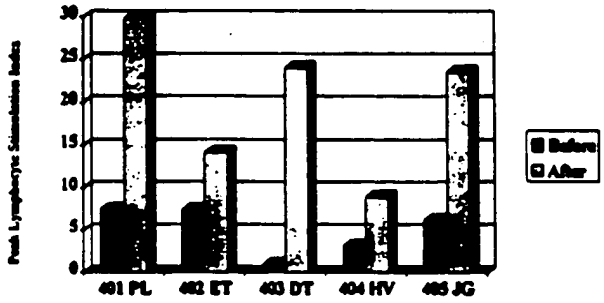
OncoVax-P Emulsion
Skin Test Reactivity to PSA



OncoVax-P Emulsion
IgG Antibody Reactivity to PSA



OncoVax-P Emulsion
Lymphocyte Proliferation to PSA



[CANCER RESEARCH 54, 3342-3345, July 1, 1994]

Advances in Brief

Polyvalent Melanoma Cell Vaccine Induces Delayed-Type Hypersensitivity and *in Vitro* Cellular Immune Response[1]

Andreas Barth, Dave S. B. Hoon, Leland J. Foshag, J. Anne Nizze, Estela Famatiga, Edward Okun, and Donald L. Morton[2]

John Wayne Cancer Institute at Saint John's Hospital and Health Center, Santa Monica, California 90404

Abstract

Patients with melanoma metastatic to distant sites or at high risk for recurrent melanoma have been treated with a polyvalent melanoma cell vaccine (MCV) in phase II protocols. We assessed *in vivo* and *in vitro* cell-mediated responses to MCV in 163 patients who had undergone surgical resection of American Joint Committee on Cancer stage III melanoma. During the first 4 months of vaccine immunotherapy, 135 patients (83%) responded by developing a positive delayed-type hypersensitivity reaction ≥6 mm to MCV. In a mixed lymphocyte tumor cell reaction using peripheral blood lymphocytes, 35 of 42 patients (83%) showed a recall proliferative response to one or more of the three cell lines of MCV. There was a significant correlation between delayed-type hypersensitivity reaction and mixed lymphocyte tumor cell reaction ($P = 0.013$). After 4 months of MCV therapy, 8 of 11 patients had an increased mixed lymphocyte tumor cell reaction to autologous melanoma cells. During the first 4 months of vaccine therapy, 16 of 33 patients developed more than a 50% increase in cytotoxic T-cell activity against one of the cell lines of MCV. Overall survival was significantly prolonged in patients with a positive delayed-type hypersensitivity reaction ($P = 0.0054$) and/or increased cytotoxic T-cell activity ($P = 0.02$). These findings suggest that MCV induces specific T-cell responses which are correlated with clinical course; the data also suggest that some of these responses are directed against autologous melanomas and may play a major role in controlling the progression of melanoma.

Introduction

Melanoma patients with regional soft tissue or lymph node metastases (AJCC[3] stage III disease) have a high risk of recurrence and a 10-year survival rate of 15–40%, depending on the extent of nodal involvement (1). In the absence of effective adjuvant chemotherapy protocols for melanoma, many investigators are attempting to induce or augment specific T-cell responses that may control disease progression (2–8). Our recent phase II study showed that patients whose advanced melanoma was treated with repeated intradermal injections of polyvalent allogeneic MCV had a significantly prolonged overall survival compared to historic controls (5). The present report analyzes the magnitude of the T-cell response to MCV in AJCC stage III melanoma patients.

Materials and Methods

Patients. We developed MCV in 1984 and initiated our phase II trial of MCV immunotherapy on September 25, 1984. In the present report, the study population of 163 patients treated between January 1, 1985, and July 1, 1989, represented all patients who had undergone surgical resection of AJCC stage III melanoma, and were followed at least 3.5 years after beginning postoperative MCV immunotherapy. There were 99 males and 64 females; their median age was 41 years (range, 16–79). The majority (63%) of the primary lesions were on the head and neck or the trunk; the remaining 37% were on the extremities or of unknown primary site. Only 33% of the primaries were thin lesions (<1.5 mm). Before surgery, 21% of the patients had in-transit metastases and 79% had lymph node metastases (24% had 1 positive lymph node, 30% had 2–4 positive nodes, and 25% had ≥5 positive nodes). All patients were clinically free of disease after surgery, as determined by physical examination and radiographic imaging of brain, chest, abdomen, and pelvis. All had a life expectancy of more than 6 months and normal blood count, liver enzymes, and creatinine. None of the female patients was pregnant. No patient had received immuno-, chemo-, or radiation therapy within the past 30 days. Written consent for MCV immunotherapy was obtained from all patients, and the MCV protocol was approved by the Human Subjects Protection Committees of the John Wayne Cancer Institute and Saint John's Hospital and Health Center, and the UCLA Jonsson Comprehensive Cancer Center.

MCV Cell Lines and Preparation. MCV comprises three well-characterized allogeneic melanoma cell lines (M10, M24, and M101). These cell lines were established in our laboratory and selected for their content of immunogenic MAA (5). Their HLA class I types are as follows: M10 (A24,33; B35,38), M24 (A11,33; B35,62), and M101 (A2,29; B44). Their surface expression of HLA-DR antigen is <2%.

The preparation of MCV is described elsewhere (5). Briefly, cells from each line are grown in serum-free medium, harvested, and pooled (8×10^6 cells/line; 24×10^6 total cells per vaccine treatment). Individual batches of MCV are analyzed for antigen expression and screened for infectious disease contaminants. MCV is irradiated at 100–150 Gy and then cryopreserved. Immediately before administration, the vaccine is thawed and washed 3 times in sterile physiological phosphate-buffered saline.

MCV Administration. MCV therapy was initiated within 3 months after surgical removal of regional lymph node or soft tissue disease (9), using a previously described protocol (5). The vaccine was injected intradermally in axillary and inguinal regions every 2 weeks × 3, and then monthly for a year. After 1 year the interval was increased to every 3 months × 4, and then every 6 months. For the first two treatments MCV was mixed with the Tice strain of *Bacillus Calmette-Guerin* (Organon Teknika Corp., Durham, NC) (8×10^6 organisms). Some patients received immunomodulators such as cimetidine (SmithKline, Philadelphia, PA), indomethacin (Lederle, Wayne, NJ), or cyclophosphamide (Mead Johnson, Princeton, NJ) (5). Clinical and laboratory evaluations were performed at each vaccine administration; chest X-rays were repeated every 2 months for the first year and then with each vaccine administration.

DTH. Equal amounts of each MCV cell line were pooled to a total dose of 2.4×10^6 cells and administered intradermally at a remote site on the forearm. DTH was defined as the average diameter of induration after 48 h. A positive response was ≥6 mm of induration, independent of accompanying erythema. The greatest DTH during the first 4 months was used for survival analysis. Since autologous cell lines were not available during the first 16 weeks of MCV therapy, autologous DTH could not be performed.

MLTR. Cryopreserved PBL from weeks 0, 4, and 16 of MCV immunotherapy were available for testing from 42 AJCC stage III patients. These PBL were simultaneously thawed, washed, and resuspended in AIM-V culture

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[1] Supported by Grant CA 12582, National Cancer Institute, Bethesda, MD; the Cancer League of Bern, Switzerland; and the Joyce and Ben Eisenberg Foundation.

[2] To whom requests for reprints should be addressed, at John Wayne Cancer Institute, 2200 Santa Monica Blvd., Santa Monica, CA 90404.

[3] The abbreviations used are: AJCC, American Joint Committee on Cancer; MAA, melanoma-associated antigens; MCV, melanoma cell vaccine; DTH, delayed-type hypersensitivity reaction; PBL, peripheral blood lymphocytes; MLTR, mixed lymphocyte tumor cell reaction; PHA, phytohemagglutinin; CTL, cytotoxic T-lymphocytes; LU, lytic units.

medium (GIBCO, Grand Island, NY) containing 10% human AB heat-inactivated serum (Irvine Scientific, Santa Ana, CA), and stimulated at a 5:1 ratio (PBL:melanoma) with each MCV line. In eight cases, a melanoma cell line developed from the patient's biopsy specimen was available for autologous MLTR (10). Autologous MLTR was also performed by using melanoma cell lines developed from biopsy specimens of three additional patients receiving MCV after surgical resection of distant metastases (AJCC stage IV disease).

The MLTR assay was performed as described elsewhere (11). Briefly, PBL were cocultured in triplicate 96-well microplates containing 200 μl of culture medium supplemented with 20 units/ml of recombinant interleukin 2 (Cetus, Emeryville, CA). Cells were then incubated for 6 days at 37°C. Respective control cultures of PBL were assayed in medium alone and with PHA (Burroughs-Wellcome, Triangle Park, NC) at a suboptimal concentration of 0.1 μg/ml. During the last 18 h of the 6-day assay, cells were pulsed with [^{3}H]thymidine (New England Nuclear, Boston, MA) and harvested. Data were analyzed as mean cpm for triplicate measurements (SD < 15%) at each time point.

Correlation of MLTR with DTH. Since DTH represents a combined reaction against all three MCV cell lines, MLTR responses to each line were combined to create a MLTR index. To determine this index, the cpm of each cell line at week 0, 4, or 16 was divided by its cpm at week 0. The MLTR index for a specific week was the sum of these values; thus, the MLTR index was always 3 at week 0. The correlation between DTH and MLTR index was determined by linear regression analysis.

Cytotoxic T-Cells. Cryopreserved PBL obtained before treatment (week 0) and after 4 and 16 weeks of MCV therapy were simultaneously thawed, washed, and grown for 4 days in AIM-V culture medium containing 10% human AB heat-inactivated serum, 10 units/ml interleukin 2, and 0.1 μg/ml PHA. CTL-mediated lysis of MCV cell lines was assessed in a standard 4-h ^{51}Cr-release assay using three effector:tumor target cell ratios (11). Results were expressed in LU according to the formula described by Pross and Maroun (12). One lytic unit was defined as the number of effector cells required to lyse 33% (LU_{33}) of 5×10^3 target cells. CTL assays were performed early during MCV treatment, without further knowledge of the patient's clinical status. Analysis of representative stimulated lymphocyte subpopulations by flow cytometry with specific T-cell monoclonal antibodies revealed 88% CD3+ and 50% CD8+.

Survival Analysis. The overall survival curves were estimated by the Kaplan-Meier method. The log-rank test was used to determine survival differences among patient subgroups defined by DTH or cytotoxicity. All tests were two-sided. Survival time was defined as the interval between the initiation of MCV therapy and the patient's death.

Results

DTH. The mean DTH of the 163 patients increased significantly from a base line of 3.2 ± 0.4 mm (mean ± SEM) pretreatment to a maximum of 13.5 ± 1.1 mm at week 4 ($P < 0.01$), and dropped slightly to about 10 mm for the following 3 months. One hundred thirty-five patients (83%) had a maximum DTH ≥ 6 mm within 4 months of beginning MCV therapy, while the remaining 28 patients (17%) exhibited no DTH response to MCV (<6 mm). Maximum DTH was 15.3 ± 1.2 mm for responders (week 4; Fig. 1*A*) and 3.6 ± 1.1 mm for nonresponders (week 16; Fig. 1*B*). The distribution of positive lymph nodes was similar between the two groups ($P = 0.8$), as was the thickness of the primary lesion. After a median follow-up of 60 months (range, 43–93), patients with a positive DTH had a median overall survival of 52 months, compared to only 22 months in patients with no DTH response ($P = 0.0054$) (Fig. 2). Patients with a DTH ≥ 6 mm and involvement of 1, 2–4, or ≥5 nodes had 5-year survival rates of 59, 46, and 34%, respectively.

Autologous MLTR. Autologous tumor cell lines were available for 11 patients. Week 16 was selected as the evaluation point based upon the number of vaccinations ($n = 5$) and the plateau phase for DTH. After 16 weeks of active specific immunotherapy with MCV, 5 of the 11 patients demonstrated more than a 3-fold increase in proliferative response to their own melan ma (Fig. 3); three other patients showed more than a 1.5-fold increase. Of the three remaining patients with no increase, two had AJCC stage IV melanoma. The eight patients with an enhanced response also showed increased reactivity to at least one of the cell lines of MCV. The overall MLTR response of the 11 patients was 28,100 ± 8,500 cpm at week 0, significantly

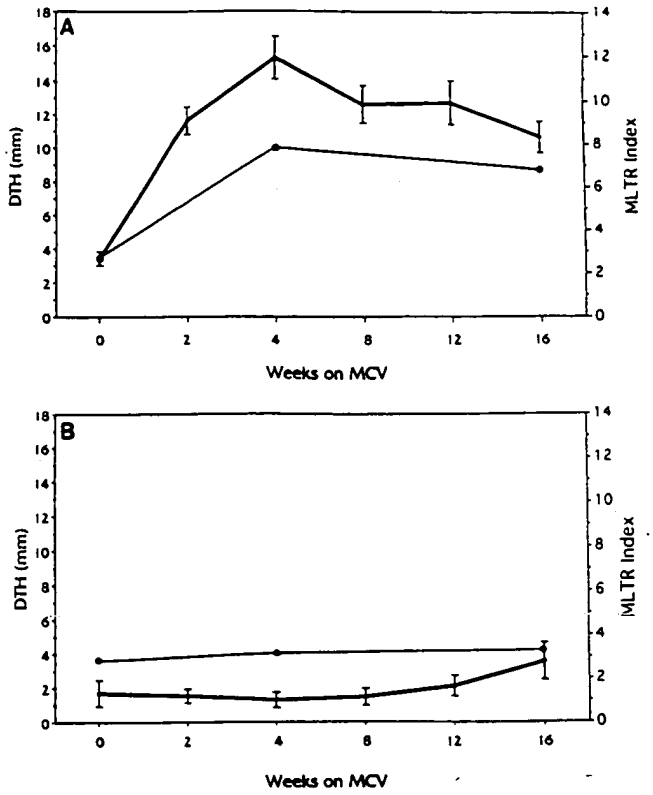

Fig. 1. *In vivo* and *in vitro* T-cell responses to MCV. ——, DTH (mean ± SEM); ——, MLTR index. *A*, DTH of 135 patients with a maximum DTH ≥ 6 mm and MLTR index of 35 responders. *B*, DTH of 28 patients with maximum DTH < 6 mm and MLTR index of 7 nonresponders. Maximum MLTR was 7.8 for responders and 3.3 for nonresponders. The magnitude of the response was independent of the number of positive lymph nodes ($P = 0.8$).

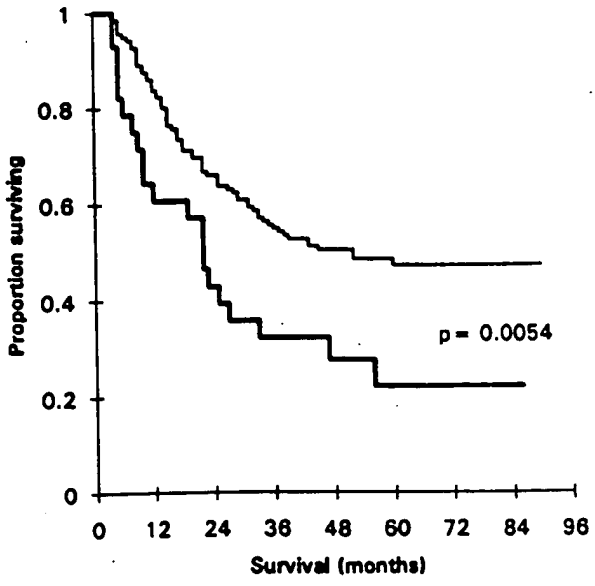

Fig. 2. Overall survival of 163 AJCC stage III melanoma patients undergoing MCV adjuvant immunotherapy initiated within 3 months after surgical removal of regional lymph nodes or soft-tissue disease. After a median follow-up of 60 months, the 135 patients with a DTH ≥ 6 mm (——) had a significantly longer overall survival than the 28 patients with DTH < 6 mm (——): median, 52 *versus* 22 months ($P = 0.0054$).

increasing to 50,000 ± 12,600 cpm at week 16 ($P < 0.01$). Stimulation of PBL with interleukin 2 alone (20 units/ml) produced a response in the 11 patients of 27,300 ± 8,200* cpm at week 0, and 32,700 ± 10,100 cpm at week 16. Similarly, stimulation with PHA alone (0.1 μg/ml) produced a response in the 11 patients of 142,500 ± 44,900 cpm at week 0, and 165,700 ± 52,400 cpm at week 16. Responses produced by stimulating PBL with either PHA alone or interleukin 2 alone failed to reach statistical significance between weeks 0 and 16.

Correlation between DTH and MLTR. PBL from weeks 0, 4, and 16 were available for 42 of the 163 patients. In 35 of 42 patients (83%), MLTR demonstrated a recall proliferative T-cell response to one or more of the three cell lines of MCV. The MLTR index of the 35 responders peaked at week 4 and then decreased slightly to a plateau (Fig. 1A); the 7 nonresponders demonstrated no change in MLTR index during the observed period (Fig. 1*B*). Linear regression analysis of data for the 42 patients confirmed a significant correlation ($P = 0.013$) between DTH and MLTR index at weeks 4 and 16.

Cytotoxic T-Cells. During the first 16 weeks, CTL-mediated lysis of one or more MCV cell lines increased in 16 of 33 patients by more than 50% above pre-MCV values, to at least 5 $LU_{33}/10^6$ effector cells (range, 5–51). CTL activity was HLA class I restricted, as demonstrated by blocking with W6/32 (anti-HLA class I) monoclonal antibody, using respective controls. Individual patients showed no significant changes (weeks 0–16) in natural killer-type activity (K562 cell killing). Fourteen of the 16 responders (88%) shared HLA-A alleles with MCV cell lines (13). Responders demonstrated a significant increase in cytotoxicity against HLA-A-matched targets from week 0 to weeks 4 and 16 ($P < 0.01$), whereas cytotoxicity of nonresponders against HLA-A-matched targets decreased slightly (Fig. 4). As demonstrated in our earlier studies (10, 11), a parallel increase in cytotoxicity against HLA-A-matched melanoma and autologous melanoma cells could be observed (Fig. 5). The overall survival of patients who had an increase in CTL activity after MCV treatment was significantly longer than that of nonresponders ($P = 0.02$), in spite of an approximately equal balance of prognostic factors (Fig. 6).

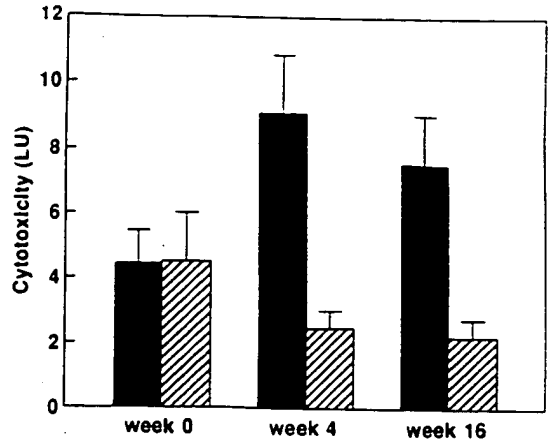

Fig. 4. Cytotoxicity of PBL (mean ± SEM) from responders (■, $n = 14$) and nonresponders (▨, $n = 11$) against allogeneic HLA-A-matched melanoma targets. Responders demonstrated a significant increase in cytotoxicity against HLA-A-matched melanoma from week 0 to weeks 4 and 16 ($P < 0.01$), whereas cytotoxicity of nonresponders against HLA-A-matched targets decreased slightly.

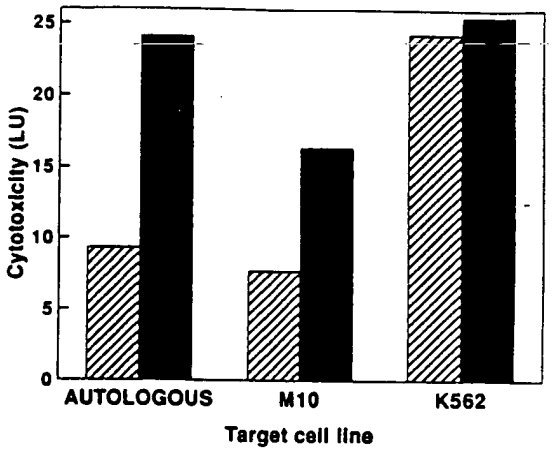

Fig. 5. Cytotoxicity of PBL (A24, 32; B7, 44) against autologous melanoma, allogeneic HLA-A-matched melanoma (M10), and K562. From week 0 (▨) to week 4 (■) there was a parallel increase in cytotoxicity against allogeneic HLA-A-matched and autologous melanoma; there was no change in cytotoxicity against K562.

Discussion

This brief report describes the induction and prognostic significance of *in vivo* and *in vitro* T-cell immunity in AJCC stage III melanoma patients receiving adjuvant MCV immunotherapy. Of 163 patients, 135 (83%) developed a DTH ≥ 6 mm following vaccine treatment. A similar degree of sensitization was demonstrated by MLTR *in vitro*. Positive DTH to MCV correlated with improved survival; by contrast, in a separate study of 148 AJCC stage IV melanoma patients (14) we were unable to demonstrate a correlation between survival and DTH to common recall antigens (mumps, *Candida albicans*, and purified protein derivative). A significant association between survival and DTH to a tumor vaccine has also been reported by Berd *et al.* (7) and Bystryn *et al.* (6) for high-risk melanoma patients, and by McCune *et al.* (15) for patients receiving active specific immunotherapy against metastatic renal cancer. Bloemena *et al.* (16) recently noted a positive correlation between DTH and *in vitro* proliferative T-cell responses to a mixture of colon tumor-associated antigens. These studies suggest that strong DTH responses during cancer vaccine therapy indicate successful activation of cell-mediated immunity.

MCV may augment T-cell responses in several ways. The enhanced T-cell response to autologous melanoma cells after MCV immunotherapy may result from direct recognition by host T-cells of MAA presented by shared or cross-reactive HLA molecules on MCV lines, as demonstrated *in vitro* (10, 13). Alternatively, activation may occur through antigen processing and presentation of MCV's MAA to host

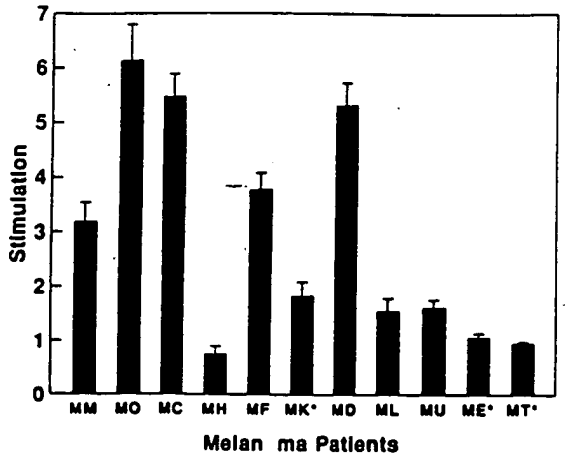

Fig. 3. Autologous MLTR (mean ± SD) of 11 patients from whose tumor a cell line was established. Results are expressed as ratios (mean cpm week 16/mean cpm week 0). By week 16 of MCV therapy, the recall proliferative response to autologous tumor had increased more than 3-fold in five patients and more than 1.5-fold in three patients. *, AJCC stage IV melanoma patient.

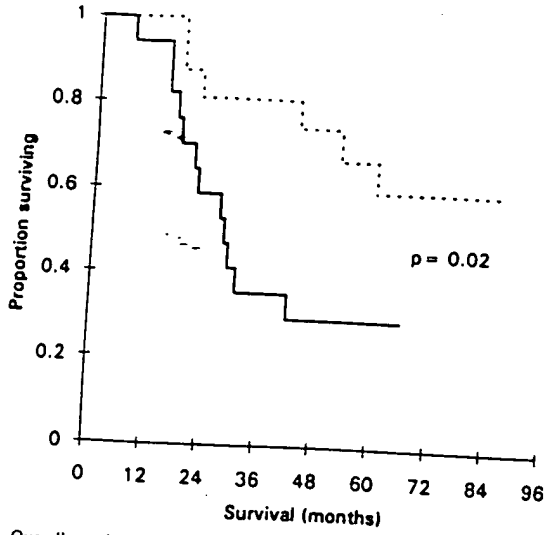

Fig. 6. Overall survival of 33 AJCC stage III melanoma patients undergoing MCV adjuvant immunotherapy initiated within 3 months after surgical removal of regional lymph nodes. After a median follow-up of 61 months, the 16 patients with more than a 50% increase in CTL activity (- - -) had a significantly longer overall survival (median, >60 months) than the 17 patients with no increase in CTL activity (median, 28 months) (——); $P = 0.02$.

T-cells by antigen-presenting cells. The allogeneic HLA antigens on the melanoma cells may stimulate alloreactive T-cells that migrate to the intradermal site of MCV injection; this induces cytokine release and attracts antigen-presenting cells in the microenvironment, which can present common MAA from vaccine cells to MAA-specific T-cells (5, 11, 17–19). This concept was recently suggested by a clinical study showing that *in vivo* transfection of the gene for an allogeneic HLA class I antigen into a patient's melanoma induced specific systemic T-cell immunity (8).

Approximately 20% of our patients did not show a T-cell response to MCV. This may reflect T-cell anergy due to continual exposure of T-cells to MAA and/or cytokine(s) (20). Alternatively, the T-cell response may have been suppressed by factors such as prostaglandins and/or suppressor T-cells (21). Specific biological modifiers administered with MCV may enhance T-cell immune responses; there is evidence that suppressor cells can be inhibited by low doses of cyclophosphamide and indomethacin, thereby up-regulating effector or helper T-cell responses (5, 21, 22).

Augmentation of the T-cell effector response to common MAA may be an important factor in the control of melanoma. In our study, enhanced CTL activity against one or more of the lines of MCV correlated with better survival. In general, CTL kill cancer cells through recognition of MAA as short peptide sequences within HLA molecules expressed on the surface of the cancer cell (23). We and others have demonstrated that CTL can kill allogeneic melanomas expressing the HLA-A antigens of CTL (11, 17, 18). We have also shown that CTL induced by sensitization with allogeneic melanoma cells expressing HLA-A antigens shared by the CTL can recognize and kill autologous melanoma cells (10). These *in vitro* studies suggest recognition of common MAA and support the potential *in vivo* mechanism of MCV cell recognition by MAA-specific T-cells. A defined polyvalent whole-cell MCV immunizes patients with multiple common MAA, thereby inducing an antigen-specific immune response that is effective against different melanoma lesions from the same patient or from different patients. Theoretically, this appears t be one of the most practical approaches t the problem of inducing active specific immunotherapy against tumors that are antigenically heterogeneous. We are currently attempting to characterize MCV antigens that are recognized by T-cells.

Acknowledgments

We thank Cindy Chang for biostatistical analysis of the data, and the clinical staff of the John Wayne Cancer Clinic.

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Cancer 1992 Mar 1;69(5):1157-1164

Relationship between immune response to melanoma vaccine immunization and clinical outcome in stage II malignant melanoma.

Bystryn JC, Oratz R, Roses D, Harris M, Henn M, Lew R

Department of Dermatology, New York University School of Medicine, NY 10016.

The authors investigated whether there was a relationship between the induction of a delayed-type hypersensitivity (DTH) response to melanoma vaccine immunization and disease recurrence. They studied prospectively 94 evaluable patients with surgically resected Stage II malignant melanoma who were immunized to a partially purified, polyvalent, melanoma antigen vaccine. The DTH response to skin tests to the vaccine was measured before treatment and at the fourth vaccine immunization. Vaccine treatment induced a strong DTH response in 29 (31%) patients, an intermediate response in 24 (25%), and no response in 41 (44%). The median disease-free survival (DFS) of patients with a strong, intermediate, and no DTH response to vaccine immunization was more than 72 months, 24 months, and 15 months, respectively. The relationship between an increase in the DTH response and a prolonged DFS was statistically significant (P = 0.02); clinically meaningful (the median DFS of patients with a strong DTH response was 4.7 years longer than that of nonresponders); and, by multivariate analysis, independent of disease severity or overall immune competence. These findings suggest, but do not prove, that vaccine treatment can slow the progression of melanoma in some patients.

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Autologous hapten-modified melanoma vaccine as postsurgical adjuvant treatment after resection of nodal metastases.

Berd D, Maguire HC Jr, Schuchter LM, Hamilton R, Hauck WW, Sato T, Mastrangelo MJ

Department of Medicine, Thomas Jefferson University, Philadelphia, PA 19107, USA.
d_berd@lac.jci.tju.edu

PURPOSE: To determine whether treatment with an autologous whole-cell vaccine modified with the hapten dinitrophenyl (DNP vaccine) is an effective postsurgical adjuvant treatment for melanoma patients with clinically evident nodal metastases. PATIENTS AND METHODS: Eligible patients had regional nodal metastases that were large enough (> or = 3 cm diameter) to prepare vaccine. Following standard lymphadenectomy, patients were treated with DNP vaccine on a monthly or weekly schedule. RESULTS: Of 62 patients with metastasis in a single lymph node bed (stage III), 36 are alive after a median follow-up time of 55 months (range, 29 to 76); the projected 5-year relapse-free and overall survival rates are 45% and 58%, respectively. Of 15 patients with metastases in two nodal sites, five are alive with a median follow-up time of 73 months. An unexpected finding was the significantly better survival of older patients; the projected 5-year survival of patients greater than 50 versus < or = 50 years was 71% and 47%, respectively (P = .011, log-rank test). The development of a positive delayed-type hypersensitivity (DTH) response to unmodified autologous melanoma cells was associated with significantly longer 5-year survival (71% v 49%; P = .031). Finally, the median survival time from date of first recurrence was significantly longer for patients whose subcutaneous recurrence exhibited an inflammatory response (> 19.4 v 5.9 months; P < .001). CONCLUSION: Postsurgical adjuvant therapy with autologous DNP-modified vaccine appears to produce survival rates that are markedly higher than have been reported with surgery alone. Moreover, this approach has some intriguing immunobiologic features that might provide insights into the human tumor-host relationship.

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Cancer Immunol Immunother 1990;**32(1)**:62-66

Renal cell carcinoma treated by vaccines for active specific immunotherapy: correlation of survival with skin testing by autologous tumor cells.

McCune CS, O'Donnell RW, Marquis DM, Sahasrabudhe DM

University of Rochester Cancer Center, University of Rochester School of Medicine and Dentistry, New York 14642.

Eighteen patients with metastatic renal cell carcinoma, who were treated by vaccines for active specific immunotherapy, also completed skin testing with autologous tumor cells, both prior to and following vaccine treatment. All patients have now been followed for more than 5 years. Ten patients who remained skin-test-negative following treatment had no clinical responses, and all had expired by 22 months. Eight patients became skin-test-positive; three of these had clinical regressions and three remain alive after more than 69 months. The survival times of the skin-test-positive group were significantly superior to those of the skin-test-negative group. The results suggest that skin testing with autologous tumor cells may accurately identify those patients who have acquired antigen-specific cell-mediated antitumor immunity.

Publication Types:

- Clinical trial

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ORIGINAL ARTICLE

Chungwen Wei · Eugene Storozynsky
Alexander J. McAdam · Kun-Yun Yeh
Brian R. Tilton · Richard A. Willis · Richard K. Barth
R. John Looney · Edith M. Lord · John G. Frelinger

Expression of human prostate-specific antigen (PSA) in a mouse tumor cell line reduces tumorigenicity and elicits PSA-specific cytotoxic T lymphocytes

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Abstract Human prostate-specific antigen (PSA) has a highly restricted tissue distribution. Its expression is essentially limited to the epithelial cells of the prostate gland. Moreover, it continues to be synthesized by prostate carcinoma cells. This makes PSA an attractive candidate for use as a target antigen in the immunotherapy of prostate cancer. As a first step in characterizing the specific immune response to PSA and its potential use as a tumor-rejection antigen, we have incorporated PSA into a well-established mouse tumor model. Line 1, a mouse lung carcinoma, and P815, a mouse mastocytoma, have been transfected with the cDNA for human PSA. Immunization with a PSA-expressing tumor cell line demonstrated a memory response to PSA which protected against subsequent challenge with PSA-expressing, but not wild-type, tumors. Tumor-infiltrating lymphocytes could be isolated from PSA-expressing tumors grown in naive hosts and were specifically cytotoxic against a syngeneic cell line that expressed PSA. Immunization with tumor cells resulted in the generation of primary and memory cytotoxic T lymphocytes (CTL) specific for PSA. The isolation of PSA-specific CTL clones from immunized animals further demonstrated that PSA can serve as a target antigen for antitumor CTL. The immunogenicity studies carried out in this mouse tumor model provide a rationale for the design of methods to elicit PSA-specific cell-mediated immunity in humans.

C. Wei · R.K. Barth · E.M. Lord · J.G. Frelinger (✉)
Cancer Center Immunology Unit, Box 704, University of Rochester, Rochester, NY 14642, USA
Fax: (716) 461-4019

E. Storozynsky · A.J. McAdam[1] · K.-Y. Yeh · B.R. Tilton · R.A. Willis · R.K. Barth · E.M. Lord · J.G. Frelinger
Department of Microbiology and Immunology, University of Rochester, Rochester, NY 14642, USA

R.J. Looney
Department of Medicine, University of Rochester, Rochester, NY 14642, USA

Present address:
[1] Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115, USA

Key words Prostate-specific antigen · Prostate adenocarcinoma · T-cell-mediated immunity · Immunotherapy

Introduction

Prostate cancer is the most frequently diagnosed malig nancy and is the second leading cause of cancer deat among American men [4–6]. If the cancer is detected earl and is localized within the prostatic capsule, it can be cure by surgery [8]. However, the prognosis is often poor i metastasis has already occurred at the time of diagnosis [6 23]. The mainstay of therapy for metastatic prostate cance is androgen ablation accomplished by either androgen antagonistic agents or castration [15]. Although androge withdrawal prolongs the period free of disease progression prostate tumor cells eventually become independent o androgen, resulting in a relapse [10, 22]. Chemotherap specifically targeted to the androgen-independent prostate tumor cells, when combined with androgen ablation, has shown little success in a series of animal studies [13, 14, 16], and there are currently no effective chemotherapeutic agents that can control the growth of androgen-independent prostate tumor cells in human [36].

Given the profound medical impact of prostate cance and the lack of adequate therapies, there is a need t develop new modalities of treatment. Immunotherapy stra tegies designed to engender a cellular immune respons have recently received much attention and represent promising approach for the treatment of many cancer [24, 32]. The benefit of this approach is that potent, long lasting immunity may be generated in the form of cytotoxi T cells (CTL) that will specifically lyse tumor cells. Such response could be effective even against metastatic cell since T cells can circulate widely in humans. Nevertheless antigen-specific immunotherapy has been hampered by th difficulty in identifying antigens capable of inducing cell mediated immunity. Tumor-specific antigens would pre sumably have to be derived from proteins expressed only i tumor cells or from mutated variants of normal proteins

These proteins are extremely rare and require laborious methods for their isolation, and in the case of mutant proteins may be different among individual cases. However, previous studies have shown that tyrosinase, a self-antigen in melanocytes, can be a target for anti-melanoma CTL [7], indicating that expression of a protein exclusively in tumor cells may not be a prerequisite for use as a tumor antigen. This may be especially true in cases such as prostate cancer in that the normal tissue from which the tumor is derived is non-essential and, therefore, may be safely ablated. This raises the possibility that prostate-specific antigen (PSA) might serve as a potential target antigen for prostate tumor immunotherapy.

PSA, a chymotrypsin-like serine protease, has a highly restricted tissue distribution and is expressed in the epithelial cells of the prostate gland, the same cell type from which most prostate tumors arise (reviewed in [34]). Indeed, PSA is widely used as a serum marker for prostate cancer [29]. Its expression is regulated by androgen, and it is present at extremely low levels in the circulation of adult men [30]. Most prostate tumors, even the poorly differentiated ones, continue to express PSA [41]. This cell-type-specific expression of PSA makes it a potential target antigen for antitumor CTL. As an initial step to examine the feasibility of eliciting a CTL response against PSA in humans, we established PSA-expressing mouse tumor cell lines, evaluated the immunogenicity of PSA expressed in the tumor cells in syngeneic mice, and analyzed the primary, memory and clonal CTL responses induced by PSA.

Materials and methods

Construction of PSA expression vector

PSA cDNA was isolated via reverse transcriptase/polymerase chain reaction [19] as follows: mRNA was isolated from the human prostate cell line LNCaP and reverse-transcribed into cDNA, which then served as the template in a polymerase chain reaction using PSA-specific oligonucleotides as primers. The sequences of the primers were

5′ primer: 5′ATCGAGTCGACGTCACCATGTGGGTCCCG3′,

3′ primer: 5′ACCTGAAGCTTTCAGGGGTTGGCCACGAT3′.

The PCR product was then subcloned into the pBluescript vector (Stratagene, LaJolla, Calif.) through the *Sal*I and *Hin*dIII sites designed in the primers. Clones were isolated and analyzed by restriction mapping and DNA sequencing. PSA cDNA was then excised from the pBluescript vector and subcloned into pHβ-Apr1-neo, a eukaryotic expression vector, at the *Sal*I and *Hin*dIII sites (pHβ-PSA).

Cell lines and PSA-expressing transfectants

Line 1, a BALB/cByJ (H-2^d) mouse lung carcinoma cell line [44], and P815, a DBA/2 (H-2^d) mastocytoma cell line [25], were grown in Excell 300 medium (JRH Biologics, Lenexa, Kan.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 2% fetal bovine serum. Line 1 and line 1/PSA cells were induced to express class I MHC by treatment with 3% dimethylsulfoxide (DMSO; Sigma, St. Louis, Mo.) for 5–20 days. To establish PSA-expressing cell lines, 2×10^5 cells were transfected with 1 μg pHβ-PSA plasmid DNA using lipofectin (BRL, Gaithersburg, Md.), and G418 (Gibco, Grand Island, N.Y.) resistant cells were cloned by limiting dilution. Clones were expanded and screened for PSA production by a sandwich enzyme-linked immunosorbent assay (ELISA).

PSA ELISA

To analyze the expression of PSA by the transfectants, 1×10^6 cells were plated and allowed to grow in 2 ml medium for 48 h before culture supernatants were harvested. PSA concentrations in the supernatants were then quantified by a sandwich ELISA using two mouse monoclonal antibodies, RLSD06 and RLSD09 (ATCC HB8527 and HB8525, Rockville, Md.), specific for human PSA. Then 96-well plates were coated with 0.1 ml RLSD06 (0.1 mg/ml in PBS) at 4 °C overnight, and washed three times with phosphate-buffered saline (PBS)/0.2% Tween 20. Plates were then blocked with Blotto-Tween at 37 °C for 1 h, and washed three times; 0.1-ml samples of twofold serially diluted culture supernatants and PSA standard (25 ng/ml, Hybritech, San Diego, Calif.) were then added to the plates and incubated at 37 °C for 1 h. After washing with PBS, 0.1 ml biotin-conjugated RLSD09 (10 μg/ml) was added and incubated for another 1 h at 37 °C. Following washes with PBS, streptavidin-conjugated horseradish peroxidase (1:500 in PBS, Jackson ImmunoResearch, West Grove, Pa.) was added. Plates were then washed and *o*-phenylenediamine (Sigma, St. Louis, Mo.) was added as the chromogen. The absorbance at 492 nm was determined using an ELISA reader.

In vivo tumor growth

Groups of 5 BALB/cByJ (BALB/c) mice (Jackson Laboratory, Bar Harbor, Me.) at 2–4 months of age were either used without immunization (unprimed) or primed twice at 10-day intervals with 5×10^6 irradiated P815/PSA or parental P815 cells i.p. and then, 10 days after boosting, challenged i.m. with 1×10^4 viable line 1 or line 1/PSA cells. Tumor-bearing legs were measured twice a week with vernier calipers, and tumor size was calculated as the square root of two perpendicular diameters. Tumors were allowed to grow until the mean thigh diameter reached 15 mm, at which point the animals were sacrificed. Animals rejecting tumors were measured for at least 100 days following tumor challenge. A χ^2 analysis was then used to determine the significance of difference in tumor incidence among experimental groups.

Isolation of tumor-infiltrating lymphocytes

Samples containing 2×10^5 line 1/PSA or parental line 1 cells in 50 μl were injected i.m. into the thighs of syngeneic BALB/c mice. Tumors were allowed to grow for 20 days before they were removed and dissociated with 10 ml 0.2% collagenase (Sigma, St. Louis, Mo.)/g tumor tissue at 37 °C for 75 min [35]. Thy1-expressing cells were enriched using sheep anti-(rat-IgG) paramagnetic beads (Dynal, Great Neck, N.Y.) coupled to T24/40.7, a rat anti-(mouse-Thy1) monoclonal antibody, as described [27, 43]. Tumor-infiltrating lymphocytes were cultured for 12–16 h in Excell medium supplemented with 5% fetal bovine serum and 10% EL4 supernatant, and then used as effector cells in the cytotoxicity assay against target cells. Culture with EL4 supernatant has been shown to have no effect on cytotoxicity (unpublished observations).

Generation of PSA-specific CTL clones

Class-I-high (DMSO-induced) and irradiated line 1/PSA or line 1 cells (1×10^7 cells/sample) were injected i.p. into BALB/c mice, which were reinjected after 2 weeks. Two weeks later, mice were sacrificed and their spleens removed. The dissociated splenocytes were restimulated for 5 days in vitro and the cytotoxicity was assayed against line 1 and P815 cells that were either untransfected or transfected with pHβ-PSA. PSA-specific CTL clones were isolated from the bulk splenocyte cultures by limiting dilution as described [3].

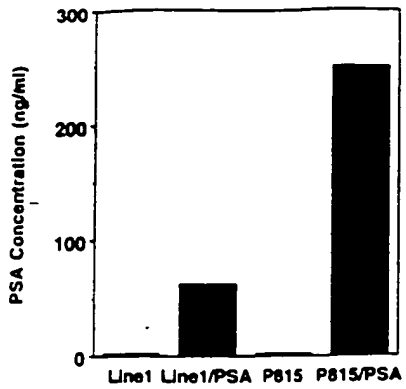

Fig. 1 Expression of prostate-specific antigen (*PSA*) in transfected line 1 and P815 cells. Samples comprising 1×10^6 cells were plated in 2 ml medium and allowed to grow for 48 h before the supernatants were collected. Twofold serially diluted culture supernatants (100 µl) from the parental or PSA-transfected line 1 and P815 cells and PSA standard (25 ng/ml at 1:1 dilution) were assayed by a sandwich enzyme-linked immunosorbent assay using mAb RLSD06 and RLSD09 as described in Materials and methods. By comparison to the PSA standard, the absorbances (at 492 nm) were then converted to concentrations (ng/ml)

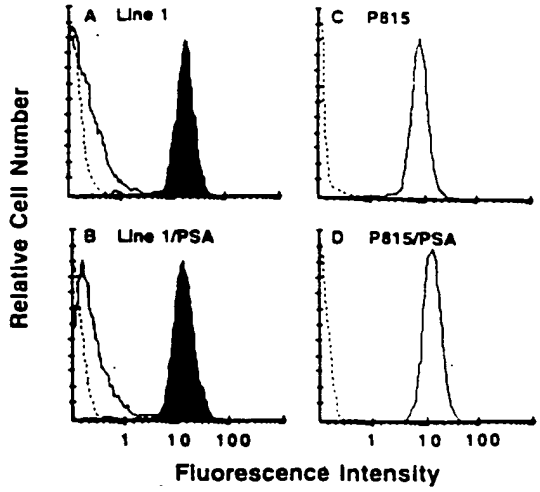

Fig. 2A–D Effect of transfection and expression of PSA on class I expression. (A) Line 1 and (B) line 1/PSA cells, either untreated or treated with 3% dimethylsulfoxide (DMSO) for 7 days, along with (C) P815 and (D) P815/PSA cells were stained for class I using AF4-62.4 (anti-H-2D^d), followed by fluorescein-isothiocyanate-conjugated goat anti-(mouse IgG) as described previously [35]. - - - Staining with the second-step antibody only, *white areas* staining for class I (uninduced), *shaded areas* class I (DMSO-induced)

^{51}Cr-release cytotoxicity assay

Six-hour cytotoxicity assays against ^{51}Cr-labeled target cells (2000/well) were performed at various effector-to-target ratios as previously described [26]. The percentage specific lysis was calculated as [(experimental release – spontaneous release) / (maximal release – spontaneous release)] × 100. Spontaneous release was never more than 20% of the maximal release for any target.

Results

Generation of PSA-expressing line 1 and P815 tumor cell lines

In order to determine whether PSA can elicit specific T-cell-mediated immunity in mice, two different mouse tumor cell lines of the same haplotype that express PSA were established. The use of two unrelated, PSA-expressing cell lines allows us to elicit responses with one line and to test the effectors on the other in order to ensure that any cytotoxic response is PSA-specific. Line 1 lung carcinoma and P815 mastocytoma cells (H-2^d) were transfected with the pHβ-Apr1-neo expression vector harboring the human PSA cDNA, and G418 resistant cells were cloned by limiting dilution. The expression of PSA in the isolated clones was analyzed by determining the PSA concentrations in the culture supernatants by ELISA. By comparison to the PSA standard, it has been shown that both the isolated PSA-transfected line 1 and P815 clones, but not the parental lines, were actively synthesizing and secreting PSA equivalent to about 150 ng/10^6 cells and 500 ng/10^6 cells respectively in a 48-h period (Fig. 1). The levels of PSA expression in these cell lines are comparable to what has been observed in the dihydrotestosterone-induced human prostate cell line LNCaP [11, 21], and are somewhat higher than that seen in the previously reported PSA-transfected mouse colon adenocarcinoma cell line MC-38 [17].

Since we wished to examine the specific cytotoxic T cell response to PSA, it was essential to measure the levels of class I MHC to ensure that expression of the transgene did not alter the class I expression in either of these PSA-transfected tumor cell lines. As shown previously, line 1 cells normally express very low levels of class I molecules. However, the expression of class I in line 1 cells can be up-regulated 30- to 50-fold by growth in vivo or by treatment with either DMSO or interferon γ in vitro [1, 28]. As expected, PSA-transfected line 1 cells (line 1/PSA) also expressed low levels of class I, and treatment with DMSO induced class I expression to a level comparable to that of DMSO-induced line 1 cells (Fig. 2A, B). It was also shown that parental P815 and PSA-transfected P815 cells (P815/PSA) had similar constitutive levels of class I expression (Fig. 2C, D). Hence, transfection with PSA did not alter the class I expression in either cell line, nor the inducibility of class I expression in line 1/PSA cells.

Priming with PSA-expressing tumor cells induces specific tumor rejection

To assess the immunogenicity of PSA expressed in the mouse tumor cells, we examined the ability of syngeneic BALB/c mice to reject PSA-expressing tumors with or without relevant priming. By immunizing mice with one tumor cell line and challenging with a viable, unrelated tumor cell line of the same haplotype, the effects of PSA expression on tumor rejection can be determined in the absence of background caused by an antitumor response.

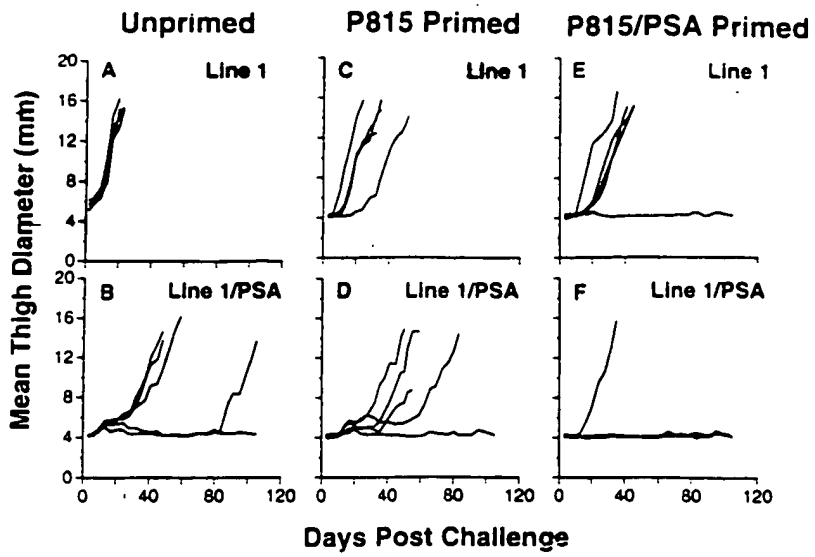

Fig. 3A–F Effect of PSA expression by the tumor cells used for priming on subsequent tumor challenges. Groups of 5 BALB/c mice were either unprimed (A, B) or primed twice with P815 (C, D) or P815/PSA cells (E, F), then challenged with 10^4 line 1 (A, C, E) or line 1/PSA (B, D, F) cells. Tumor growth was measured as described in Materials and methods. Each line represents the mean thigh diameter of a single mouse

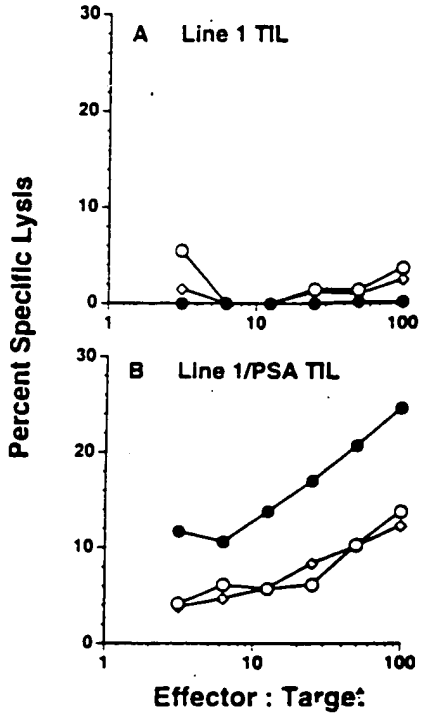

Fig. 4A,B Characterization of tumor-infiltrating lymphocytes isolated from line 1 and line 1/PSA tumors. Tumor-infiltrating lymphocytes isolated from mice inoculated with (A) line 1 (line 1 tumor-infiltrating lymphocytes, TIL) or (B) line 1/PSA (line 1/PSA TIL) were tested in a 6-h cytotoxicity assay against DMSO-induced line 1 (◇), line 1/PSA (○) and DMSO-induced line 1/PSA (●) cells

Naive animals and animals immunized with irradiated P815 or P815/PSA cells were challenged 10 days later with viable line 1 or line 1/PSA cells. In unprimed and P815-primed control animals, tumors formed in all the animals challenged with 10^4 parental line 1 cells, and in four out of five animals challenged with the same dose of line 1/PSA cells (one mouse with tumor died prematurely at day 55 in the P815-primed group, Fig. 3A–D). As expected, immunization with P815/PSA offered no protection against a subsequent challenge with 10^4 parental line 1 cells, as tumors grew in four out of five animals (Fig. 3E). However, priming with P815/PSA provided significant protection against subsequent challenges with 10^4 line 1/PSA cells. As shown in Fig. 3F, four out of five animals primed with P815/PSA did not grow line 1/PSA tumors. A χ^2 analysis revealed that this protection was statistically significant ($P < 0.05$) when compared to the other three groups of mice (Fig. 3C–E), strongly suggesting that tumor rejection was mediated through a PSA-specific immune response.

The line 1/PSA tumor that grew in a P815/PSA-primed animal was harvested and cultured in vitro and the supernatant from this culture was tested in the PSA sandwich ELISA. PSA was not detected in the culture supernatant, indicating that this tumor no longer expressed PSA. We also found that, in some cases, the growth of line 1/PSA tumors in P815-primed animals resulted in a cell line that no longer expressed PSA (data not shown). Whether this is due to selection of a negative variant in vivo or to another form of antigenic variation related to the instability of the transfected gene is not clear.

PSA-expressing tumors induce a PSA-specific CTL response in unimmunized mice

Line 1/PSA tumors appeared to establish later than the parental line 1 tumors in both naive animals (Fig. 3A, B) and mice primed with P815 (Fig. 3C, D). This was not due to an intrinsic difference in the growth rates of the cells, as both line 1 and line 1/PSA exhibited the same growth rates in vitro with a doubling time of approximately 13 h (data

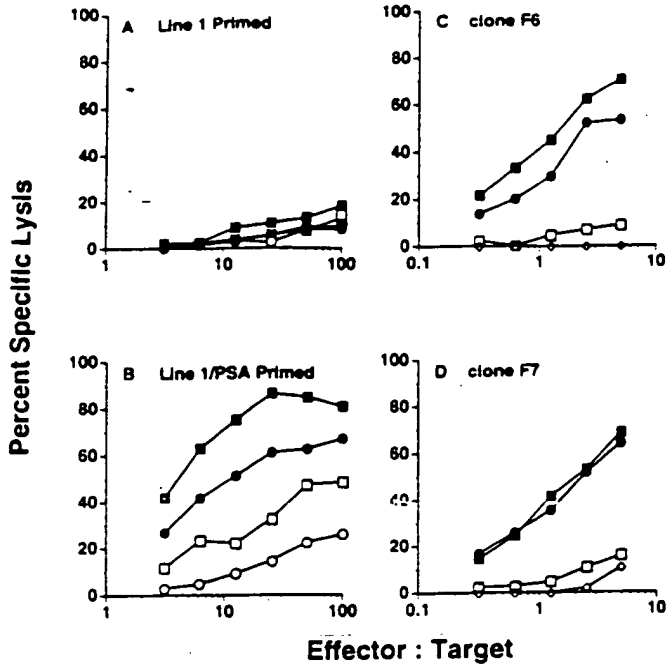

Fig. 5 Characterization of splenocytes and isolation of cytotoxic T lymphocyte (CTL) clones specific for PSA. Splenocytes isolated from mice immunized with (A) line 1 or (B) line 1/PSA, and (C, D) two CTL clones isolated from the bulk splenocyte cultures were tested in a 6-h cytotoxicity assay against DMSO-induced line 1 (◇), line 1/PSA (○), DMSO-induced line 1/PSA (●), P815 (□) and P815/PSA (■) cells

not shown). Since transfection of line 1 cells with PSA did not change the inducibility of class I expression in vitro (Fig. 2A, B) or in vivo (data not shown), the retarded growth of line 1/PSA cells in naive and P815-primed animals suggested the induction of a primary anti-PSA immune response. To analyze directly whether such a primary response against PSA was occurring, naive syngeneic BALB/c mice were inoculated with line 1/PSA or parental line 1 cells and tumors were allowed to grow. Tumor-infiltrating lymphocytes (TIL) were harvested 20 days after implantation, and the cytolytic activity of the TIL was determined by 6-h ^{51}Cr-release assays against DMSO-induced line 1/PSA (class-I-high), line 1/PSA (class-I-low) and parental DMSO-induced line 1 (class-I-high) cells. The inducibility of class I expression in line 1/PSA cells provides an internal control for whether the PSA-induced immune response is due to class-I-restricted CTL. As shown in Fig. 4, TIL isolated from line 1/PSA tumors, but not from parental line 1 tumors, contained PSA-specific CTL. The cytolytic activity of the PSA-specific CTL depended on the expression of class I molecules as well as PSA, as neither uninduced line 1/PSA nor DMSO-induced line 1 cells could serve as targets (Fig. 4). These results indicate that PSA elicits a specific, class-I-restricted primary CTL response in mice. The generation of a primary CTL response against PSA may, in part, cause the delay in growth of line 1/PSA tumors in naive and P815-primed animals as observed in Fig. 3.

Immunization with a PSA-expressing tumor results in PSA-specific memory CTL

The observation that expression of PSA in the primi tumor elicited rejection of subsequent challenges with PS expressing tumors as well as the demonstration of TIL growing, PSA-expressing tumors prompted us to exami the memory response against PSA. BALB/c mice we primed twice with irradiated line 1 or line 1/PSA, ar splenocytes were removed and re-stimulated again in vit before their cytolytic activity was tested against target cel Splenocytes isolated from mice immunized with line 1 ce showed no cytolytic activity against PSA-expressing ce (Fig. 5A). However, splenocytes from line 1/PSA-imm nized mice lysed target cells in a PSA-dependent mann As shown in Fig. 5B, these splenocytes were capable lysing DMSO-induced line 1/PSA targets but not li 1/PSA (class-I-low) targets or DMSO-induced line 1 targe (data not shown), demonstrating that the effectors we specific for PSA in a class-I-restricted fashion. Specifici for PSA was further shown by demonstrating that unrelated syngeneic target cell, P815/PSA, could be lys efficiently by these effectors while the parental line, P81 was lysed to a much lesser extent.

Although most of the lysis appeared to be PSA-specifi there were also significant levels of PSA-independe cytolytic activity in the bulk splenocyte cultures. To e amine the specificity of the memory CTL response again PSA, the bulk splenocyte cultures were cloned by limitir dilution and PSA-specific CTL clones were isolated on t basis of their high lysis of PSA-expressing target cells. Tl specific cytolytic activity for PSA-expressing targets of tv of these clones is shown in Fig. 5C, D. These clon showed highly efficient lysis of DMSO-induced line PSA and P815/PSA targets, which express both PSA ar class I, while there was no specific lysis of the class- expressing targets DMSO-induced line 1 and P815. Tl data from these cytotoxicity assays demonstrate the pre ence of a classical, class-I-restricted memory CTL respons to PSA in immunized animals.

Discussion

Immunotherapy for prostate tumors designed to promc cell-mediated immunity has recently been pursued. It h been shown that interleukin-2-transfected Dunning r prostate carcinoma cells induce a local antitumor respon in the rat model, which appears to be due to a non-T-ce mediated mechanism [31]. In the same Dunning rat mod granulocyte/macrophage-colony-stimulating-factor-trans- duced tumor vaccine cells have been shown to prolo survival in rats with established prostate tumor deposited a distant site [38]. However, these reports neither chara terized the antigens recognized nor dem nstrated directly T-cell-mediated immune response. A recent report, which a PSA-transfected mouse colon adenocarcinon cell line was shown to grow faster in athymic mice th

in syngeneic C57BL/6 mice, suggested the possible involvement of T cells in slowing the growth of PSA-expressing tumors [17]. However, the T cell response was not further characterized. Finally, a PSA-expressing vaccinia vector has recently been constructed in an effort to generate a PSA vaccine that could potentially be used in humans [12]. Inoculation of rhesus monkeys with this vaccinia vector resulted in a PSA-specific IgM response as well as T cell proliferation in response to PSA. Again, however, there was no further characterization of T cell subsets involved or whether the vaccine could engender an antitumor response.

In this report, we extended these studies by showing that the expression of human PSA in mouse tumor cells elicits a potent CTL response in mice. Immunization with a PSA-expressing tumor resulted in rejection of subsequent challenges with tumors that expressed PSA. The generation of PSA-specific CTL clones clearly is an indication of the ability of PSA to act as a target for antitumor CTL. Interestingly, the expression of a single foreign protein is sufficient to engender a potent T cell response and protect against an aggressive and poorly immunogenic tumor. The immunogenicity studies presented here thus serve as a basis for the design of methods to elicit a PSA-specific CTL response in humans. If a PSA-specific CTL response can be elicited in patients with prostate cancer, then an immune response could be directed to the tumor.

One obvious obstacle in eliciting an immune response to PSA is that humans are generally tolerant to self-antigens. However, the large number of tissue-specific autoimmune diseases in humans demonstrate that self-tolerance is not always absolute. Moreover, recent studies on human melanoma have shown that tyrosinase, Melan-A-MART-1, gp100 and gp75, all normal self-proteins specific to the melanocyte lineage, can serve as targets for anti-melanoma CTL [2, 7, 18, 42]. The existence of anti-melanoma CTL specific for self-proteins clearly demonstrates that self-antigens can be targets for CTL.

PSA offers an excellent opportunity to examine the ability of self-antigens to serve as targets for CTL. Antigens that are present at low levels or sequestered from the immune system [39] enhance the likelihood of unresponsiveness. Since PSA is highly restricted in its tissue expression and is expressed late in development and at low levels in circulation, it may be below the detection limit of the immune system. Furthermore, it has recently become apparent that sequestration need not be absolute. It may be sufficient that the target antigens are not presented in an immunogenic fashion. This phenomenon, termed clonal ignorance, has been experimentally demonstrated in elegant transgenic experiments using a lymphocytic choriomeningitis virus (LCMV) protein expressed in pancreatic islet cells [33]. These cells were tolerated until the LCMV protein was introduced in an immunogenic form by infecting the mice with LCMV, inducing T cell infiltration into pancreatic islets with resultant hypoglycemia. These findings suggest that the transgenic mice were not tolerant to the LCMV protein but merely ignorant of it. It is possible that non-responsiveness to PSA in humans might be similar. Indeed, the detection of anti-PSA antibodies in patients with advanced prostate cancer [9], in which PSA expression is greatly increased, indicates that PSA can be the target of a humoral autoimmune response and that tolerance to PSA is not absolute. Since CD4 cells are required for efficient antibody production, this finding also suggests the presence of a CD4 T cell response.

There are also theoretical reasons why tolerance may not be absolute. While tolerance may be induced for certain epitopes of self-antigens, it is possible that there are other determinants present in the same self-antigen that have not induced self-tolerance (reviewed in [20, 32, 40]). In essence the immune system is ignorant of these "cryptic" epitopes. However, under certain circumstances, these epitopes can be displayed in an immunogenic form and induce an autoimmune response. For instance, it has been shown that binding of HIV gp120 to CD4 causes extensive internalization of this complex, which leads to increased presentation of CD4 epitopes on the MHC molecules of that cell [37]. This altered presentation leads to an increase in the presentation of a cryptic CD4 epitope and subsequent autoimmune-T-cell-mediated destruction of these cells. Therefore, even if humans are truly tolerant of the dominant antigenic peptides of PSA, the unresponsiveness to PSA as a whole may be reversed by presenting the cryptic determinants of PSA in an immunogenic form.

We are currently developing a transgenic mouse model in which human PSA is specifically expressed in the prostate gland. This model more closely approximates the situation in humans in that PSA becomes a self-antigen. By immunizing these mice with PSA-expressing tumor cells or by other methods, we will be able to evaluate the effect of endogenous PSA expression on the immune response to PSA. In our current work, we have shown that a strong immune response can be effective against tumors expressing PSA. Future studies with our transgenic mouse model will allow us to develop methods for generating an autoimmune, cell-mediated response against PSA that will be translatable to the immunotherapy of human prostate cancer.

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Phase I Clinical Trial: T-Cell Therapy for Prostate Cancer Using Autologous Dendritic Cells Pulsed With HLA-A0201-Specific Peptides from Prostate-Specific Membrane Antigen

G. Murphy, B. Tjoa, H. Ragde, G. Kenny, and A. Boynton

Pacific Northwest Cancer Foundation, Cancer Research Division (G.M., B.T., H.R., G.K.), Department of Molecular Medicine (A.B.), Northwest Hospital, Seattle, Washington

BACKGROUND. Conventional treatment for metastatic prostate cancer have failed to demonstrate curative potential in all patients. Investigations involving the role of T-cell immunity in the clearance of neoplastic cells are now available. Development of T-cell immunotherapy may give a new approach to the treatment of advanced metastatic prostate cancer.
METHODS. A phase I clinical trial assessing the administration of autologous dendritic cells (DC) pulsed with HLA-A0201-specific prostate-specific membrane antigen (PSMA) peptides were conducted. Participants were divided into five groups receiving four or five infusions of peptides alone (PSM-P1 or PSM-P2; groups 1 and 2, respectively), autologous DC (group 3), or DC pulsed with PSM-P1 or P2 (groups 4 and 5, respectively).
RESULTS. No significant toxicity was observed in all five groups. Cellular response against PSM-P1 and -P2 was observed in HLA-$A2^+$ patients infused with DC pulsed with PSM-P1 or -P2 (groups 4 and 5), respectively. An average decrease in PSA was detected only in group 5. Seven partial responders were identified based on NPCP criteria + PSA.
CONCLUSIONS. Infusions of test substances were well tolerated by all study participants. Detection of cellular response and decrease in PSA level in some patients who received DC pulsed with PSM-P2 indicate this method's potential in prostate cancer therapy.

KEY WORDS: **dendritic cells (DC), T cell, immunotherapy, prostate cancer, Phase I trial**

INTRODUCTION

Immunotherapy has recently emerged as a promising new approach in cancer therapy, in light of new advances in two important components. Discovery of cancer-specific antigens (Ag), e.g., melanoma, breast, and ovarian cancer-specific/associated protein and peptides have provided the first component: targets for specific therapy [1–5]. T cells recognize processed peptide antigens bound to the major histocompatibility complex (MHC) proteins [6]. Previous studies have identified specific motifs of MHC class I-binding peptides [7–10]. These advances are useful for predicting potential T-cell epitopes from proteins of known amino acid sequences. HLA-A0201 (a subset of HLA-A2 family) is one of the MHC class I protein expressed by a large proportion of the population [11]. Using the specific motif, we identified HLA-A0201-specific prostate-specific membrane antigen (PSMA) peptides for use as Ag in our prostate cancer immunotherapy study [12]. PSMA is a specific prostate antigen recognized by monoclonal antibody 7E11.C5 [13,14]. Levels of PSMA are elevated in the serum of hormone refractory advanced prostate cancer patients. PSMA is also found in normal male serum and seminal fluid, and in prostate epithelial cells [15,16].

Address reprint requests to Dr. Gerald P. Murphy, Pacific Northwest Cancer Foundation, Northwest Hospital, 120 Northgate Plaza, Suite 205, Seattle, WA 98125.

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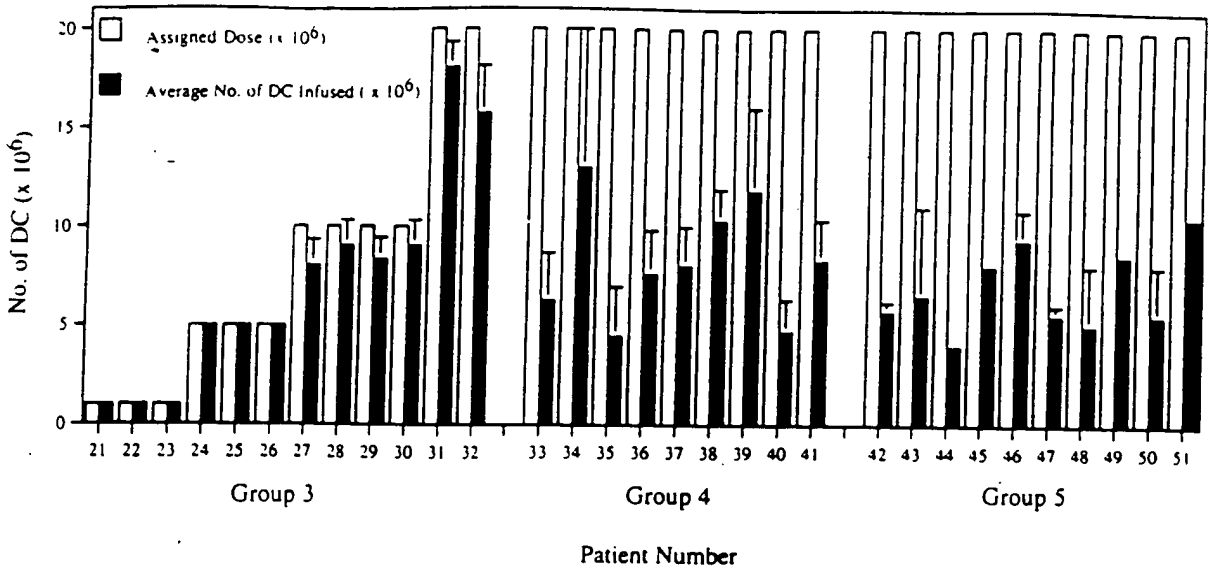

Fig. 1. Number of dendritic cells (DC) infused in this study. Three groups (groups 3–5) were infused with autologous DC cultured from individual patients' peripheral blood mononuclear cell population. Group 3 patients were divided into subgroups which received the assigned dose of 1, 5, 10, or 20 × 10^6 DC. Groups 4 and 5 received the assigned dose of up to 2 × 10^7 DC. The actual number of cells infused depended on the number of DC harvested from culture before each infusions. The assigned dose, and the average number of DC actually infused [and standard error of the mean (SEM)] for each patient are depicted using white and black bars respectively. The average number of cell infused in groups 4 and 5 are 8.2 × 10^6 and 7.3 × 10^6, respectively.

TABLE I. Phase I Patient Prostate Cancer Staging*

Group	No. of patients	Stage				
		B2*	C*	D0*	D1*	D2
1	11	0	0	1	1	9
2	9	0	2	0	0	7
3	12	0	1	0	1	10
4	9	1	0	0	1	7
5	10	0	0	3	1	6
All	51	1	3	4	4	39

*Patients in these stages also had elevations in PSA serum levels.

The second component was the requirement of efficient presentation of cancer Ag by the host's antigen presenting cells (APC) to circulating T cells in the generation of an effective anticancer response [17]. Dendritic cells (DC) are known as professional APC and have an exceptional ability to present Ag to naive $CD4^+$ and $CD8^+$ T cells both in vivo and in vitro [18,19]. DC are present in very small numbers in many tissues of lymphoid and nonlymphoid origin [19]. Several investigators, including our laboratory, have developed protocols to generate large numbers of DC from peripheral blood or bone marrow [20–24]. We further reported the ability to culture DC from peripheral blood of advanced prostate cancer patients, many of whom with impaired immune system due to previous radiation, chemo-, or hormone therapy [20]. DC cultured from these patients pulsed with a number of prostate-specific Ag, including two HLA-A0201-specific PSMA peptides (designated PSM-P1 and PSM-P2), have the capacity to activate naive autologous T cells in vitro [12].

Based on the in vitro study, we conducted a phase I clinical trial to assess the safe administration of autologous DC pulsed with HLA-A0201-specific PSMA peptides to patients with advanced metastatic, hormone resistant prostate cancer. Patients were divided into five treatment arms that receive four or five infusions of PSM-P1 or PSM-P2 alone, autologous DC alone, or autologous DC pulsed with PSM-P1 or PSM-P2. Findings from this study support the potentiality of DC-based prostate cancer vaccines.

MATERIALS AND METHODS

Reagents and Cytokines

PSMA peptides with HLA-A0201-specific motif (PSM-P1: LLHETDSAV; PSM-P2: ALFDIESKV) were synthesized and purified (>95% purity) by Peninsula Laboratories (Belmont, CA) and obtained as a lyophilized powder. The powder was dissolved in 0.9% saline (USP 0.9%, sodium chloride injection, Ameri-

TABLE II. Clinical Profiles of Phase I Clinical Trial Participants*

Group	No. of Patients	Hormone refractory	Postprostatectomy	Postradiation therapy	Postorchiectomy	HLA-A2+	(+) Skin tests 0	1	2	3
1	11	11	5	8	5	7	2	5	1	3
2	9	9	2	3	6	5	4	1	2	2
3	12	12	2	2	6	10	3	4	2	3
4	9	9	3	7	5	3	3	4	1	1
5	10	10	4	8	4	5	2	4	1	3
All	51	51	16	28	26	30	14	18	7	12

*HLA-A2 expression was determined in our laboratory as described under Materials and Methods. Three types of skin test were conducted: diphtheria/tetanus, streptokinase, and mumps antigens.

TABLE III. Toxicity Observations in Phase I Study: Occurences of Mild Hypotension* Observed During Infusions

Infusion No.	Group 1	Group 2	Group 3	Group 4	Group 5	Total
1	5	2	5	4	3	19
2	0	1	1	1	0	3
3	4	1	3	1	0	9
4	1	0	1	0	ND†	2

*Average systolic/diastolic over 4 hr <30/20 mm Hg (e.g., 136/89–116/69). All blood pressure decreases returned to pretreatment levels.

†Data not available at the time of this analysis.

can Reagent Laboratories, Shirley, NY) to a concentration of 2 mg/ml. The peptide solution was sterilized using a 0.2-μm filtration. Sterile 0.9% saline was added to achieve concentrations of 200, 20, and 2 μg/ml; 100-μl aliquots of the three peptide concentrations containing 20, 2, or 0.2 μg peptide were transferred into sterile containers and frozen until use. Granulocyte-macrophage colony-stimulating factor (GM-CSF) approved for human use was provided by Immunex (Seattle, WA). Interleukin-4 (IL-4) approved for human use was provided by Schering-Plough Research Institute (Kenilworth, NY).

DC Culture

In this investigation, 50–100 ml peripheral blood was drawn and peripheral blood mononuclear cells (PBMC) isolated using Lymphoprep (GIBCO-BRL, Gaithersburg, MD) density-gradient centrifugation. PBMC were resuspended in complete medium (OPTIMEM medium [GIBCO-BRL, Gaithersburg, MD] and 5% heat-inactivated autologous plasma) and plated in a 75-cm^2 tissue culture flask (2–3 × 10^7 cells/flask). Cell suspensions were incubated in a humidified incubator (37°C, 5% CO_2) for 60 min. Non-adherent cells were removed and adherent cells were washed gently with warm (37°C) complete medium. Dendritic cell propagation medium (DCPM: complete medium, 1,000 U/ml GM-CSF and 1,000 U/ml IL-4) was added to the adherent cells (10 ml/flask). These cells were cultured for 4–6 days.

Patient Population

Fifty-one patients with advanced hormone-resistant prostate cancer were selected for the study, which included a signed informed consent. Their prostate cancer staging, previous treatment history, and pre-study delayed-type hypersensitivity (DTH) skin test results are shown in Tables I and II. In addition, patients were tested for expression of HLA-A2 expression using flow cytometric analysis with a monoclonal antibody specific for HLA-A2 (BB7.2 [25]), as well as polymerase chain reaction (PCR) (Table II). During the period of observation of 6–12 months, 50–100 ml of heparinized peripheral blood was drawn for DC culture and immunological monitoring.

Treatment Groups

Participants were divided into five treatment groups. In the first group, the patients were infused with PSM-P1 (LLHETDSAV) peptide at 0.2, 2.0, and 20.0 μg; group 2 received PSM-P2 peptide (ALFDIESKV) at the same concentrations received by group 1; group 3 received autologous DC at 1, 5, 10, or 20 × 10^6 per infusion; group 4 and 5 received up to 2 × 10^7 autologous DC pulsed for 2 hr with 1 μg/ml PSM-P1 or PSM-P2, respectively. Every group received four or five doses of the test substance at 6–8 week intervals during the study period.

Infusions

PSMA peptides. One frozen aliquot of PSM-P1 or -P2 containing the appropriate amount of peptide

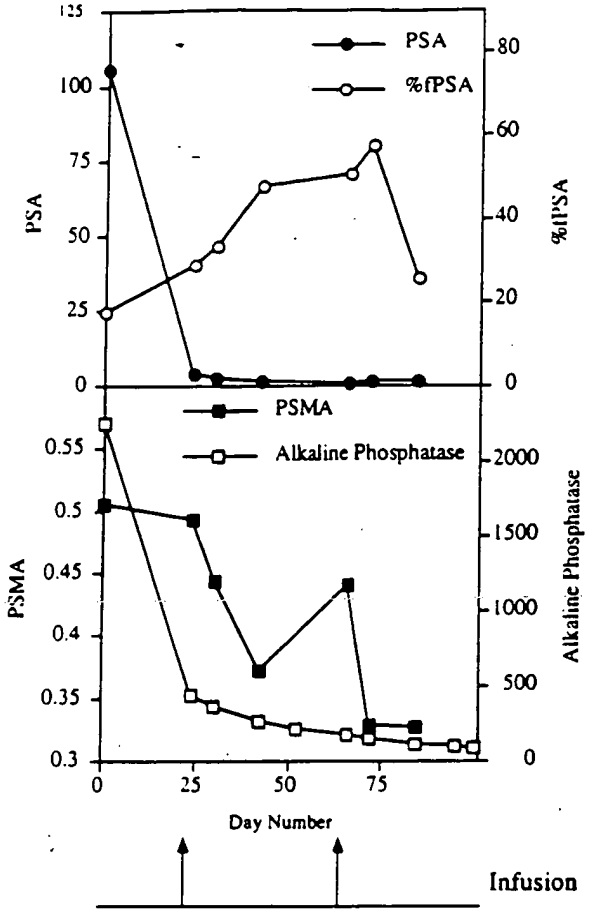

Fig. 2. Partial responder who had external radiation before vaccine therapy. This patient was admitted to this study with a history of bony metastasis (stage D_2) and underwent palliative radiation therapy to his right shoulder and lower spine 3 weeks before the first infusion. He is an HLA-A2 positive individual enrolled in group 5. He was infused with 5 and 6 million autologous DC pulsed with PSM-P2 in the first two infusion cycles. The T-cell response to PSM-P2 was modulated after infusions (stimulation ratios postinfusion 1 = 1.9 and postinfusion 2 = 4.7). *Top:* Decrease in PSA and increase in % free PSA levels. *Bottom:* Decrease in PSMA and alkaline phosphatase levels during the course of the clinical trial. Arrows along the horizontal axis indicate the date of DC infusions.

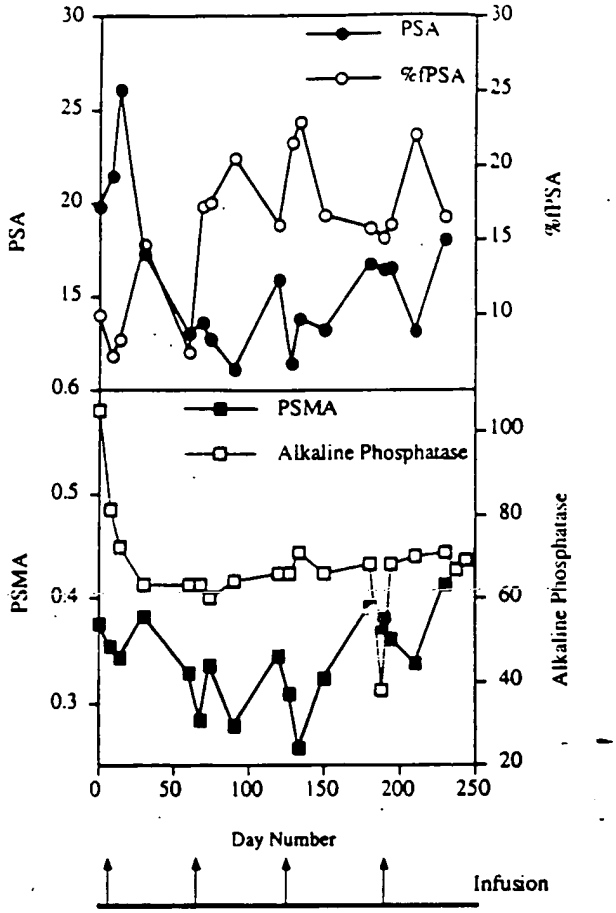

Fig. 3. Partial responder from group I who was in remission for 25–225 Days, and is now in progression.

(20, 2, or 0.2 μg) was thawed and transferred into single-use 10-ml injectible USP 0.9% saline vials (American Reagent Laboratories). The peptide solution was infused over 5 min into the lateral entry port of a fast-flowing intravenous infusion of 0.9% saline.

DC/Peptide-Pulsed DC. Cells were washed three times with 0.9% saline to eliminate trace medium, counted, and transferred to into a single-use 10-ml injectible 0.9% saline. DC were infused over 30 min with a total volume of 100 ml 0.9% saline.

Toxicity Monitoring

Both acute and chronic toxicity were monitored. Monitoring for acute toxicity took place during and immediately following infusion for a period of 1–4 hr. Patients were observed for the development of (1) allergic reaction and (2) anaphylactic reaction during that time. Chronic toxicity were evaluated at periodic examinations.

Cytokine Level Monitoring

Serum tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-α) from peripheral blood drawn one day post-infusion were measured using enzyme-linked immunoabsorbent assay (ELISA) kits (BioSource International, Camarillo, CA).

Clinical Monitoring

Patients were followed before during and after treatment with periodic PSA (prostate specific antigen; Tandem-E PSA kit, Hybritech, La Jolla, CA), free

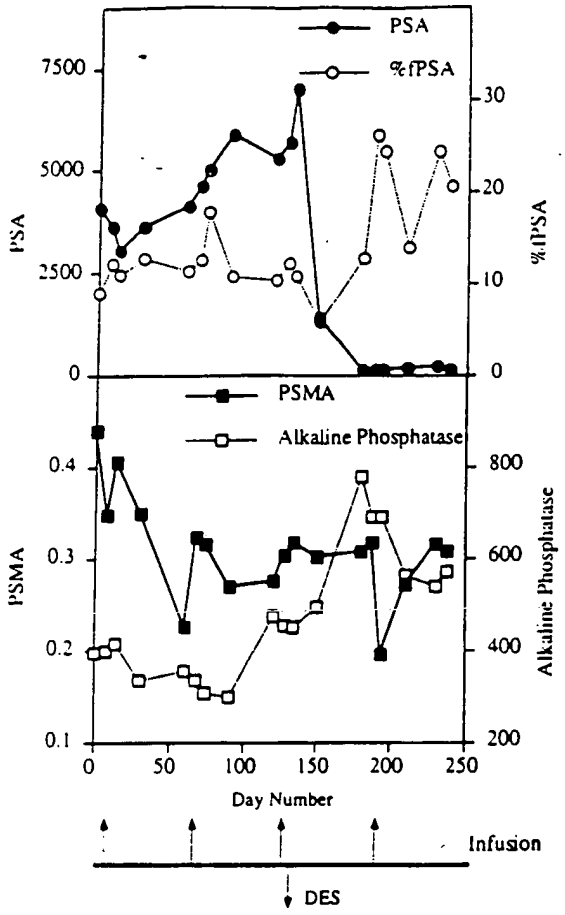

Fig. 4. Partial responder from group 2 who, at the time of the response, had a change in hormone therapy to DES.

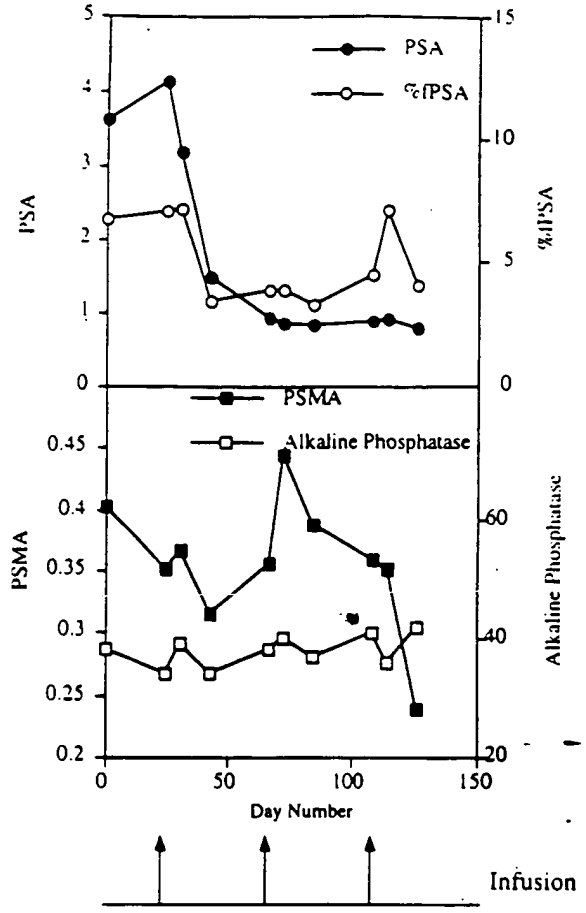

Fig. 5. Partial responder from group 4 in Whom the % free PSA remains low at present.

PSA (Tandem-R PSA kit, Hybritech), PSMA, complete blood counts (CBCs), CHEM-22, initial chest radiography, bone scan, and computed tomography (CT) scan of the pelvis. All testing was conducted on either an inpatient or outpatient basis at Northwest Hospital. Patients were also evaluated every infusion cycle by one of the study physicians.

Immunological Monitoring

T-cell lymphoproliferative responses upon presentation of PSM-P1 or -P2 to patients' peripheral blood lymphocytes. In short, preinfusion and 7-day postinfusion PBMC freeze store vials were thawed, washed, cells counted, and viability assessed and resuspended at 2×10^6 cells/ml; 200,000 cells per well were incubated in 96-well microtiter plates in triplicate. PSM-P1 or -P2 (10 μg/ml) were added at the start of the culture. On day 5, 1 μCi tritiated thymidine (^{3}H-TdR) were added to each well. Cells were further incubated for 24 hr and harvested, and the tritiated thymidine incorporated was measured using a liquid scintillation counter. Stimulation ratio was calculated as the average cpm from postinfusion wells divided by the average cpm from preinfusion wells.

RESULTS

Patient Population

Fifty-one patients with hormone-refractory prostate cancer were selected for this study (Tables I, II). Most (47/51) have metastatic disease, most of whom (39/47) are stage D2 patients. A large proportion of the population have undergone previous radiation therapy (28/51) and/or surgical procedures, such as prostatectomy (16/51), orchiectomy (26/51), or a combination of treatments. Most study participants have impaired cellular immunity, as less than 25% (12/51) of the population responded to all three antigens in a DTH test conducted prior to the start of the study (Table II). More than 50% (30/51) of the patients in this study express HLA-A2 (Table II).

Study participants were divided into five treat-

ment groups as described under Materials and Methods. In short, patients in the first group received PSM-P1 at 0.2, 2.0, and 20.0 μg; group 2 received PSM-P2 at the same concentrations as group 1; group 3 received autologous DC at 1, 5 10, or 20 × 10^6 per infusion; and groups 4 and 5 received up to 2 × 10^7 DC pulsed with PSM-P1 or -P2, respectively. The number of DC infused in groups 4 and 5 varied between 2 × 10^6 to 2 × 10^7 cells, depending on the number of cells cultured from patients' blood. Figure 1 shows the average number of DC infused to individual patients in groups 3–5. Patients in groups 1–3 have completed at least four infusions. Patients in groups 4 and 5 have completed at least three and two infusions, respectively.

Toxicity Monitoring

Acute toxicity monitored during and immediately following infusion include the development of allergic and anaphylactic reactions. The only significant reaction observed was that of a mild to moderate hypotension (without associated pulse rate change, following the infusion in all groups (24/51 subjects). The incidence was highest at the first infusion (Table III) and declined thereafter. A given subject experiencing hypotension at the first infusion did not experience repeat hypotension on follow-up infusions in most cases (16/51 subjects did not experience repeat hypotension; 8/51 did, with 7 subjects experiencing hypotension in 2/4 infusions, and one subject experiencing hypotension in 3/4 infusions). Blood pressure reductions did not persists beyond 4 hr postinfusion. All subjects were recumbent during this observation period. No sequelae were noted. In addition, 3/51 patients reported a feeling of fatigue postinfusion that lasted 7–8 days. No significant rise in serum TNF-α or IFN-γ was observed.

Clinical and Immunological Monitoring

CBC and SMA-12 studies, as well as prostate marker studies were conducted at every interval. In addition, patients were also monitored for cellular immune modulation to the appropriate PSMA peptides (PSM-P1 or -P2). The results of these studies are summarized in Tables IV and V. Owing to the large variation of pretreatment levels of PSA among the patients, each treatment group was divided into two categories: low (initial PSA values of 0–10) and high (initial PSA values >10) PSA subgroups. Analysis of the five treatment groups a whole demonstrated a modest increase in PSA levels in group 1–3 patients, who received peptide or DC alone, and group 4 patients, who received DC pulsed with PSM-P1, with high initial PSA. No significant change in PSA levels

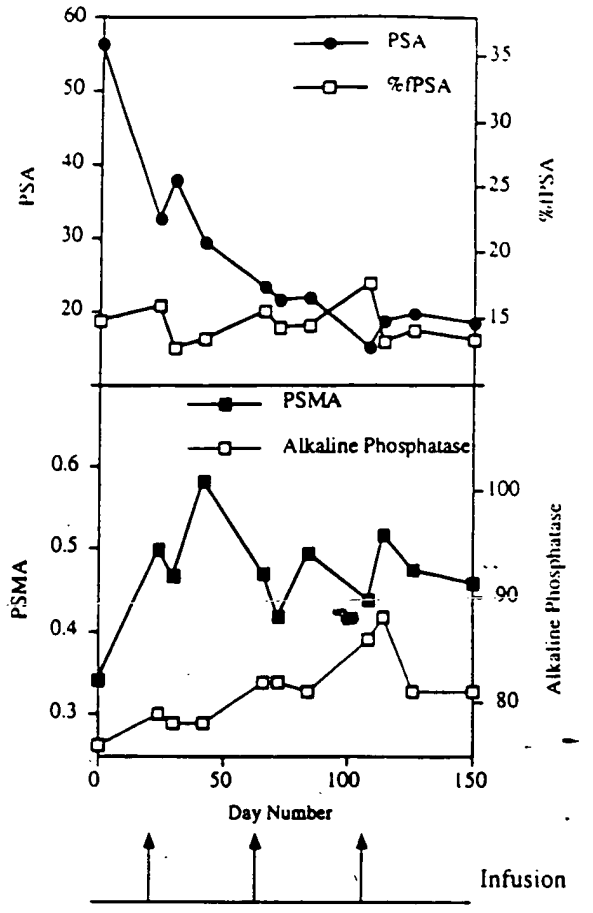

Fig. 6. Partial responder from group 4 in whom the PSMA values remain high.

was observed in group 4 patients with low initial PSA, as well as patients in group 5 (who received DC pulsed with PSM-P2; Table IV). In addition, no significant change was observed in the levels of absolute lymphocyte, hematocrit and alkaline phosphatase (Table V).

Analysis of cellular immune modulation as analyzed using an in vitro proliferation assay within each treatment group as a whole showed no significant response in groups 1–4 and a modest increase in group 5. However, a higher extent of cellular response are observed within the HLA-A2-positive subpopulation of groups 4 and 5 (Table VI), with an average stimulation ratio of 1.9 and 2.8 after two infusions of DC pulsed with PSM-P1 or -P2, respectively. Analyses of HLA-A2-positive populations within groups 1–2 (infused with peptide alone) or group 3 (DC alone) showed no significant increase in stimulation ratio. Furthermore, analyses of prostate markers within the HLA-A2-positive populations showed a decrease in PSA and a modest increase in

TABLE IV. Summary of Prostate Marker Levels[a]

Group[a]	Subgroup	Category	Preinfusion Mean	Preinfusion SEM	Postinfusion 1 Mean	Postinfusion 1 SEM	Postinfusion 2 Mean	Postinfusion 2 SEM	Postinfusion 3 Mean	Postinfusion 3 SEM
1	Low	PSA	1.71	0.76	1.81	0.76	2.72	1.19	8.63	4.87
PSM-P1	PSA	%fPSA	10.8	5.6	16.3	8.5	15.9	8.6	10.9	6
	[5]	PSMA	0.24	0.04	0.24	0.05	0.3	0.03	0.34	0.05
	High	PSA	3.23	120	399	156	109	46	142	41
	PSA	%fPSA	18.4	7.2	15.4	4.7	21.2	1.7	18.1	2.5
	[6]	PSMA	0.25	0.06	0.29	0.04	0.28	0.02	0.28	0.03
2	Low	PSA	2.77	1.5	4.15	2.41	2.78	1.81	4.96	3.49
PSM-P2	PSA	%fPSA	5.6	3.6	4.3	2.3	6.3	3.5	6	2.7
	[5]	PSMA	0.33	0.04	0.4	0.04	0.36	0.02	0.38	0.04
	High	PSA	1,099	994	862	728	1,369	1,211	2,891	1,812
	PSA	%fPSA	22	5.8	19.6	6.1	22.7	6.2	17.1	6
	[4]	PSMA	0.27	0.09	0.25	0.08	0.26	0.05	0.32	0.02
3	Low	PSA	7.24	1.81	8.41	3.2	11.4	5.66	10.5	4.16
Autologous DC	PSA	%fPSA	10.3	4.3	14.4	7.7	13.7	6	11.8	4.6
	[5]	PSMA	0.29	0.02	0.29	0.04	0.29	0.04	0.28	0.01
	High	PSA	128	43	196	58	462	163	580	241
	PSA	%fPSA	27.8	6.4	23.6	6	21.1	5	24.3	6.5
	[7]	PSMA	0.33	0.04	0.29	0.04	0.38	0.04	0.4	0.05
4	Low	PSA	2.39	0.93	1.91	0.91	1.27	0.63	2.08	1.2
DC/PSM-P1	PSA	%fPSA	13.5	8.5	15.1	8.8	17.7	11.1	15.8	7.7
	[5]	PSMA	0.37	0.04	0.39	0.05	0.39	0.03	0.34	0.02
	High	PSA	89	24	148	0.14	213	110	211	100
	PSA	%fPSA	16.4	2	13.9	0.5	12.4	0.7	15.2	0.9
	[4]	PSMA	0.31	0.02	0.33	0.05	0.38	0.06	0.39	0.06
5	Low	PSA	3.34	0.83	2.6	0.3	3.5	0.47	—	—
DC/PSM-P2	PSA	%fPSA	10.9	4	14.2	4.8	14.1	0.2	—	—
	[5]	PSMA	0.35	0.05	0.36	0.05	0.35	0.04	—	—
	High	PSA	57.4	20.5	33	18	50.8	33.9	—	—
	PSA	%fPSA	15.2	3.6	19.2	5.1	26.2	9.9	—	—
	[4]	PSMA	0.38	0.04	0.34	0.04	0.31	0.03	—	—

[a]Each group is divided into low (initial PSA values 0–10) and high PSA (initial PSA values >10) categories. Normal ranges for the prostate markers: PSA = (0–4 ng/ml); %fPSA = (17.86 ± 1.89); PSMA = (0.08–0.21) (ref. 26). Mean and standard error of the mean (SEM) values are obtained from patient sera obtained preinfusion and 7 days postinfusion.
[a]The number of patients in each subgroup is shown in parentheses.

percentage free PSA (%fPSA) levels only within patients in group 5 (Table VI).

Responders

Individual analyses of patients' clinical response based on National Prostate Cancer Project (NPCP) criteria + PSA are presented in Table VII. Figure 2 reports the prostate markers and alkaline phosphatase level of one of the partial responders. This patient was admitted to this study with a history of bony metastasis (stage D_2) and underwent palliative radiation therapy to his right shoulder and lower LS spine 3 weeks before the first infusion. He is an HLA-A2 positive individual enrolled in group 5 (DC pulsed with PSM-P2 infusion). Immunological monitoring conducted showed increase in T-cell response to PSM-P2 peptide with each infusion (stimulation ratios postinfusion 1 = 1.9 and postinfusion 2 = 4.7) (Fig. 2). His PSA level decreased from an initial value of 105.51 to 3.54 after the first infusion cycle and stayed stable at 0.53–1.08 over 11 weeks of observation. In addition, decreasing levels of PSMA (from 0.506 to 0.326) and alkaline phosphatase (from 2,245 to 116), and an increase of %fPSA (17.2 to 57.4) were observed. Furthermore, a bone scan conducted after

TABLE V. Clinical Monitoring Summary[a]

Group[a]	Category	Preinfusion		Postinfusion 1		Postinfusion 2		Postinfusion 3		Postinfusion 4	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	Stimulation ratio[b]	—	—	1.09	0.03	1.59	0.3	1.33	0.17	—	—
[11]	Absolute lymphocyte	852	159	690	122	852	85	729	105	509	53
	Hematocrit	35.6	1.2	34.9	1.5	35.8	1.9	35.6	1.7	33	1.5
	Alkaline phosphatase	152	39.8	184	60	136	31	145	36	140	23
2	Stimulation ratio[b]	—	—	1.22	0.17	1.32	0.13	1.26	0.2	—	—
[9]	Absolute lymphocyte[c]	1,440	340	1,296	283	1,298	330	1,189	26	1,660	268
	Hematocrit[d]	36.6	1.8	35	1.7	35.8	2.3	34.5	2	36.2	2.1
	Alkaline phosphatase[e]	127	37	126	37	112	26	144	44	137	48
3	Stimulation ratio[b]	—	—	1.13	0.06	1.2	0.06	1.23	0.07	—	—
[12]	Absolute lymphocyte[c]	1,322	82	1,341	134	1,292	103	1,345	129	—	—
	Hematocrit[d]	38.6	1.9	37.6	1.9	36.5	2.1	37.6	2.2	—	—
	Alkaline phosphatase[e]	145	30	179	51	293	104	371	160	—	—
4	Stimulation ratio[b]	—	—	1.87	0.34	1.37	0.21	1.2	0.12	—	—
[9]	Absolute lymphocyte[c]	833	144	838	129	800	76	1,037	143	—	—
	Hematocrit[d]	37	0.9	36.9	0.8	37	0.5	38.3	0.6	—	—
	Alkaline phosphatase[e]	67	9	69	11	73	11	79	16	—	—
5	Stimulation ratio[b]	—	—	1.33	0.07	2.01	0.42	—	—	—	—
[10]	Absolute lymphocyte[c]	1,155	202	1,026	178	972	162	—	—	—	—
	Hematocrit[d]	37.8	1.5	37.8	1.1	37.4	1	—	—	—	—
	Alkaline phosphatase[e]	323	225	111	146	103	12.5	—	—	—	—

[a]Mean and standard error of the mean (SEM) values are obtained from patient sera obtained preinfusion and 7 days postinfusion.
[a]The number of patients in each group is shown in parentheses.
[b]Measure of T-cell lymphoproliferative response against the appropriate PSMA peptide as described under Materials and Methods.
[c]Normal range: 1,000–3,500 × 10^3 ml.
[d]Normal range: 40–52%.
[e]Normal range: 25–125 IU/L.

two infusion cycles revealed a considerable improvement with decreased activity seen throughout the skeletal metastasis compared to two previous examinations conducted before his enrollment in the study and after one infusion cycle.

Figure 3 illustrates a partial responder from group I. The response lasted 25–225 days and was of short duration. A bone scan has confirmed the progression. Figure 4 shows a partial responder from group 2. The hormone treatment was changed to 1 mgm/qd D.E.S. Thus, the origin of the response is not clear. As shown in Figure 5, a responder in group 4 did not show a resolution of all four markers (PSA, %fPSA, alkaline phosphatase, and PSMA). The %fPSA was not restored to normal ranges, and the normal alkaline phosphatase remained such. By contrast, as shown in Figure 6, another group 4 responder had no response in terms of the PSMA levels. For the determination of a response, the NPCP criteria plus PSA reduction >50% was required. The additional markers are under comparative study. Thus, to date, seven partial responders have been identified along with 29 progression, 11 stable, and 4 nonevaluable cases.

DISCUSSION

In this study, we have examined the safe administration of HLA-A0201-specific PSMA peptides (PSM-P1 and -P2), autologous DC, and PSM-P1 and -P2 pulsed autologous DC to 51 patients with advanced hormone-refractory prostate cancer, most of whom (39/51) are in stage D_2. Many of these patients are anemic and have undergone various treatments that have resulted in an impaired immune competency. Less than 25% of this population were considered fully immunocompetent at the start of the study, as assessed by DTH skin tests. Almost one-third of these patients failed to respond to any of the three test antigens.

At the completion of up to four cycles of infusion,

TABLE VI. PSA Levels and T-Cell Response Against PSMA Peptide in HLA-A2-Positive Patients[a]

Group[a]	Category	Preinfusion Ave	Preinfusion SEM	Postinfusion 1 Ave	Postinfusion 1 SEM	Postinfusion 2 Ave	Postinfusion 2 SEM	Postinfusion 3 Ave	Postinfusion 3 SEM
1	Stimulation ratio[b]	—	—	1.01	0.1	1.39	0.31	1.16	0.18
	PSA	4.47	3.17	5.53	4.23	4.01	2.03	9.24	4.18
[7]	% freePSA	10.5	5.17	16.1	7.62	18.9	7.29	13.6	5.6
2	Stimulation ratio[b]	—	—	1	0.07	1.1	0.05	1.3	0.25
	PSA	875	896	684	661	1086	1095	2146	1.656
[5]	% freePSA	17.3	7.55	15.8	7.48	19.6	7.7	14.3	6.8
4	Stimulation ratio[b]	—	—	2.2	0.29	1.9	0.21	—	—
	PSA	74.1	38.9	93.3	51.3	177.7	132	211.6	145.8
[3]	% freePSA	26	10.5	26.5	11	30.9	14.2	29.6	10.4
5	Stimulation ratio[b]	—	—	1.6	0.07	2.8	0.43	—	—
	PSA	31.8	21.1	10.35	5.28	12.1	6.23	—	—
[6]	% freePSA	15.8	4.46	20.7	5.87	23	11.8	—	—

[a]The number of HLA-A2-positive patients in each group is shown in parentheses.
[b]Measure of T-cell lymphoproliferative response against the appropriate PSMA peptide depicting the ratio of postinfusion and preinfusion values.

TABLE VII. Summary of Phase I Trial Clinical Status[a]

Group	Pro-gression	Stable	Partial responder	Non-evaluable	Total	HLA-A2 +/−
1	7	3	1	0	11	7/4
2	5	3	1	0	9	5/4
3	10	2	0	0	12	11/12
4	3	2	4	0	9	3/6
5	7	1	1	1[b]	10	6/3

[a]Clinical status was determined as of 9/16/96 using NPCP criteria + PSA.
[b]One patient died before receiving any infusion.

maximum tolerated dose was not achieved, as no significant acute or chronic toxicity was observed in all doses of test substances. Elevation of the level of serum TNF-α and IFN-γ, which are usually associated with immune response-mediated toxicity, were absent in all study groups [27]. The only effect observed were mild to moderate cases of hypotension, presumably due to the length of infusion and observation period immediately following infusion, in which patients were recumbent. Blood pressure drop did not persist over 4 hr postinfusion.

Data from immunological monitoring studies show an increase of T-cell response to the appropriate PSMA peptides in HLA-A2-positive patients in groups 4 and 5. These patients received infusions of autologous DC pulsed with PSM-P1 or -P2, respectively, implying specific in vivo presentation of the PSMA peptides with HLA-A0201-specific motif, by autologous DC to circulating T cells. No significant responses were observed in group 3 patients, who received autologous DC alone, as well as group 1 and 2 patients, who received peptides alone. These results demonstrate the requirement of both components in the generation of an effective immune response.

PSA assay is the only Food and Drug Administration (FDA)-approved clinical assay for monitoring and early detection of prostate cancer to date. HLA-A2-positive patients in group 5 exhibited a significant reduction in average PSA values, presumably due to lysis of prostate cancer cells by PSM-P2-specific autologous T cells. Based on the NPCP criteria + PSA, seven partial responders were identified. Only one of the partial responders was identified from group 5 after two cycles of infusions. Four non-evaluable patients are all from group 5, two of whom are HLA-A2 positive and will continue to receive infusions and clinical evaluations for the rest of the study period.

The seven responders have exhibited responses of 150–225 days duration to date. These are relatively short. In five of these seven patients, all four markers were changed, viz. total PSA, % free PSA, PSMA, and total alkaline phosphatase. These are being further studied. We will soon also test the direct measurement of bone alkaline phosphatase isozymes. In follow-up evaluation, additional responses may be identified. At present we feel justified in planning a phase II study which will include DC pulsed with PSMA or PSMA peptides.

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